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Low-Dose Radiation-Induced Enhancement of Thymic Lymphomagenesis in Lck-Bax Mice is Dependent on LET and Gender

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Abstract

The hypothesis that mitochondrial dysfunction and increased superoxide levels in thymocytes over expressing Bax (Lck-Bax1 and Lck-Bax38&1) contributes to lymphomagenesis after low-dose radiation was tested. Lck-Bax1 single-transgenic and Lck-Bax38&1 double-transgenic mice were exposed to single whole-body doses of 10 or 100 cGy of ¹³⁷Cs or iron ions (1,000 MeV/n, 150 keV/μm) or silicon ions (300 MeV/n, 67 keV/μm). A 10 cGy dose of ¹³⁷Cs significantly increased the incidence and onset of thymic lymphomas in female Lck-Bax1 mice. In Lck-Bax38&1 mice, a 100 cGy dose of high-LET iron ions caused a significant dose dependent acceleration of lymphomagenesis in both males and females that was not seen with silicon ions. To determine the contribution of mitochondrial oxidative metabolism, Lck-Bax38&1 over expressing mice were crossed with knockouts of the mitochondrial protein deacetylase, Sirtuin 3 (Sirt3), which regulates superoxide metabolism. Sirt3^{-/-}/Lck-Bax38&1 mice demonstrated significant increases in thymocyte superoxide levels and acceleration of lymphomagenesis ($P < 0.001$). These results show that lymphomagenesis in Bax over expressing animals is enhanced by radiation exposure in both an LET and gender dependent fashion. These findings support the hypothesis that mitochondrial dysfunction leads to increased superoxide levels and accelerates lymphomagenesis in Lck-Bax transgenic mice.

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INTRODUCTION

The free radical theory of aging first proposed by Dr. Denman Harmon in the 1950's hypothesized that "...the phenomenon of growth, decline, and death..." known as aging was driven by free radical-based damage to biomolecules (1). This theory was based on the assumption that the detrimental effects seen in organisms after exposure to ionizing radiation were mediated by the same oxygen free radicals that arise naturally during oxidative metabolism to cause aging (1). In support of this hypothesis, radio-protective compounds (reduced thiols) administered continuously increased average lifespan by 20–35% in unirradiated AKR and C3H mice by delaying the onset of specific tumors to which these mice are prone (2, 3). Taken together these studies highlighted a common free-radical based mechanism between metabolic aging and radiation-induced damage, and suggest that metabolic defects may impact both aging and radiation response. The current study uses a mouse model of thymocyte-specific mitochondrial dysfunction caused by Bax overexpression that leads to enhanced thymic lymphoma development (4) to address the hypothesis that preexisting metabolic defects leading to increased steady-state levels of superoxide amplify the carcinogenic effects of low-dose radiation exposure.

Radiation-induced oxidative damage involves the immediate formation of free radicals from primary ionization events that trigger a cascade of oxidant species formation from metabolic processes that persists long after exposure (5). While the overall energy deposition at the time of exposure is small, radiation-induced ionization events produce both direct damage to biomolecules and indirect effects through radiolysis of H₂O and reactive oxygen species (ROS) generation. Numerous intracellular biological mechanisms have evolved to counteract the damaging effects of ROS and ionizing radiation, such as induction of antioxidant enzymes that directly scavenge hydrogen peroxide and superoxide, which can act as both damaging agents and signaling molecules integral to radiation-induced carcinogenesis (6, 7).

Ionizing radiation exposure, through damage to DNA, acts as an initiating stress in the classical three stage model of carcinogenesis consisting of initiation, promotion and progression (8). Superoxide, in contrast, is a weak initiating agent but acts as a strong promoting agent as demonstrated previously where superoxide dismutase inhibited tumor promotion in mouse skin chemical carcinogenesis studies (9). We propose that irradiation of Lck-Bax mice that already have increased steady-state levels of superoxide and are lymphoma-prone, with a single exposure to low- or high-LET radiation should yield insight into the role of oxidative metabolism in mechanisms underlying low-dose radiation-induced carcinogenesis.

Numerous epidemiological studies performed over the past five decades from cohorts of atomic bomb survivors, radiation workers and miners conclusively demonstrate an increased risk of cancer from exposure to low-linear energy transfer (LET) ionizing radiation (10). While this link between radiation exposures >50 cGy and increased cancer risk has been determined, extrapolating high-dose data to make predictions of risk for very low-dose exposures is difficult (11). The recent combined disasters at Fukushima and the continued refinement of radiation protection standards reinforce the importance of understanding how low-dose radiation exposures modulate lifetime cancer risk, and the mechanisms underlying the disease process and individual susceptibility. According to the Life Span Study (LSS) of atomic bomb survivors, women have a twofold increased excess relative risk for all solid tumors, which increases to fourfold when considering only lung cancer, compared to men (10). Lymphomagenesis, on the other hand, has only recently been associated with doses of low-LET radiation below 1 Gy (12). This newly discovered late effect, evident 35 years after exposure, is significant in the wake of earlier studies showing no association between

low-dose radiation exposure and lymphoma (13). As the effects of low-dose ionizing radiation require a longer time interval to become evident, biological factors such as gender could become increasingly important.

Epidemiological data regarding more densely ionizing radiation is limited to a small cohort of cancer patients treated with high-LET proton and carbon ion therapy at specialized facilities, as well as astronauts exposed to galactic cosmic rays (GCR) during spaceflight. Due to the relatively small number of astronauts with spaceflight in the last fifty years, estimates of cancer risks involved while conducting future long-duration missions have been derived from large cohorts of persons exposed to low-LET ionizing radiation. Strikingly, this analysis recommended 20–30% lower dose limits for female astronauts, highlighting gender-specific radiation-induced cancer risks in human populations (14).

GCR are comprised of protons and high-charge and energy (HZE) particles from elements such as silicon, neon, titanium and iron. HZE particles pose an unavoidable hazard outside Earth's protective magnetic field as they are capable of passing directly through the light materials used in spacecraft and depositing a dense ionization track of clustered damage as they traverse the human body (15). It is estimated that each cell in an astronaut's body will be traversed by an HZE nuclei every few months, and the long term health effects from extended HZE-particle exposures represent one of the most significant unknown risk factors in multi-year space missions, such as the exploration of other planets in our solar system (16). In addition, the lack of epidemiological data regarding multi-year exposures to high-LET GCR places the impetus on laboratory studies to discover the tissue-specific biological risks associated with each dose and type of GCR, and to elucidate critical pathways involved in HZE-particle-induced health effects (16).

The biological effects of both low- and high-LET ionizing radiation have been shown to involve a critical free radical component, as radio-protective compounds that scavenge free radicals (such as amifostine) are effective at inhibiting radiation damage after exposure to both low- and high-LET radiation (17). In addition, many radio-protective compounds including thiols, that induce superoxide and hydrogen peroxide scavenging enzymes, ultimately derive their radio-protective effects from increased ROS metabolism (17, 18). These findings have led to the emerging concept that even the direct ionization of biomolecules by high-LET radiation requires the persistent generation of reactive species by metabolic processes to mediate the full biological effect after exposure.

Alterations in mitochondrial metabolism and increases in oxidant generation are recognized as key events in cellular injury and transformation processes after radiation exposure (19). Recently, it has been demonstrated that a mutant form of succinate dehydrogenase (SDH) subunit C sensitizes cells in culture to low doses of low-LET ionizing radiation (20). To examine the effects of mitochondrial oxidative metabolism in low-dose ionizing radiation from both low-LET and high-LET sources, a Lck-Bax mouse model of mitochondrial dysfunction, with increased levels of super-oxide production in thymocytes, was selected for study (4).

C57B/6 Lck-Bax transgenic mice demonstrate enforced Bax overexpression mediated through transgenic insertion of extra copies of the mitochondrial-localized Bax gene under the control of the lymphocyte-specific protein tyrosine kinase promoter (Lck) and represent a murine model of thymocyte-specific lymphoma development (4). Lck-Bax38&1 thymocytes also demonstrate increased levels of superoxide and it was previously shown that loss of one SOD2 allele accelerated lymphomagenesis by approximately six weeks, showing that steady-state levels of mitochondrial superoxide played an integral role in lymphoma development (4). To test the role of mitochondrial dysfunction leading to

increased superoxide levels in radiation responses *in vivo*, Lck-Bax1 and Lck-Bax38&1 mice were treated with single whole-body doses of low-LET ^{137}Cs or densely ionizing HZE iron or silicon ions and thymic lymphoma development was monitored. In addition the Lck-Bax38&1 mice were crossed with C57B/6 mice lacking the mitochondrial protein deacetylase, Sirt3, which has been shown to regulate mitochondrial superoxide metabolism to confirm the involvement of mitochondrial superoxide in lymphomagenesis (21, 22). The results of these studies show that lymphoma-genesis in Bax over expressing animals is enhanced by radiation exposure in both an LET and gender dependent fashion. These findings also support the hypothesis that mitochondrial dysfunction caused by disrupting Sirt3 leads to increased superoxide levels and accelerates lymphomagenesis in Lck-Bax38&1 transgenic mice.

MATERIALS AND METHODS

Mice and Irradiation Conditions

Lck-Bax38&1 and LckBax1 transgenic mice were generated as previously described and maintained on a C57B/6 background (4, 23). Lck-Bax38&1 mice are positive for two independently generated transgenic lines as previously described (23). Lck-Bax1 is a single transgenic line that has only moderately accelerated thymic lymphomagenesis (23). Both Lck-Bax1 and Lck-Bax38&1 mice used in this study had wild-type p53 alleles (p53^{+/+}). Mice were genotyped by DNA extracted from a tail snip using PCR as previously described (23). To generate Sirt3^{-/-} Lck-Bax38&1 mice, Sirt3^{-/-} mice (National Institutes of Health mice no. 1476 with *SIR3* gene disrupted by retroviral insertion, C57B/6 background) were obtained from the NIH and genotyped using the forward Sirt3WT primer 5'-ATCTCGCA-GATAGGCTATCAGC-3' and reverse Sirt3WT primer 5'-TCTTGCAGTTGCATCCGACTTGTG-3' along with forward Sirt3-KO primer 5'-AACGATGAAATCTCCCGGTTTGGC-3' and reverse Sirt3KO primer 5'-TCTTGCAGTTGCATCCGACTTGTG-3'. Thermal cycling at 94°C (45 s), 55°C (45 s), 72°C (45 s) with 34 total cycles produces from this two primer pair scheme a 336 bp fragment in mice with a Sirt3WT allele mice and a 160 bp fragment in mice with a Sirt3^{-/-} allele. Sirt3^{-/-} mice on a C57B/6 background were crossed with Lck-Bax38&1 mice on a C57B/6 background to generate Sirt3^{+/-}/Lck-Bax38&1 and Sirt3^{-/-} mice which were then bred to produce Sirt3^{-/-}/Lck-Bax38&1. Sirt3^{-/-}/Lck-Bax38&1 and Sirt3^{-/-} breeding pairs were then used to generate Sirt3^{-/-}/Lck-Bax38&1 tumor watch cohorts, where overall survival of this novel mouse background was measured against the sham-exposed Lck-Bax38&1 (Sirt3^{+/+}) from the high- and low-LET radiation cohorts. All mouse studies were conducted under protocols approved by the University of Iowa Institutional Animal Care and Use Committee (IACUC) and Brookhaven National Laboratory (IACUC). Mice were housed in a temperature and humidity controlled environment in filter top cages with *ad libitum* access to food (NIH-31 diet, Harlan Laboratories product number 7913) and water in an SPF facility administered by the Office of Animal Research at the University of Iowa. Mice used in silicon and iron-particle radiation studies were bred at the University of Iowa, housed for approximately two weeks at the Brookhaven Laboratory Animal Facility, and returned to the University of Iowa for the duration of the study.

Low-LET ^{137}Cs irradiated cohorts were whole-body irradiated with a single dose of 10 cGy or 100 cGy by the Radiation and Free Radical Research Core laboratory at the University of Iowa using a ^{137}Cs source at a dose rate of 0.35 Gy min⁻¹ (J. L. Shephard & Associates, San Fernando, CA). Mice were irradiated in a circular Lucite wheel on a rotating base with 12 separate compartments. Sham controls were transported along with irradiated groups and subject to the same conditions. Iron and silicon cohorts were shipped to Brookhaven National Laboratory one week prior to exposure and transported to the NASA Space Radiation Laboratory (NSRL) the morning of irradiation experiments. Mice were randomly

assigned into groups and loaded into ventilated 50 mL conical tubes placed into a 10-bay foam holder and secured onto the beam line. Mice were whole-body irradiated using a 20 × 20 cm beam area with a single dose of 10 or 100 cGy of silicon or iron HZE, administered over approximately 1 min. These experiments took place during the 09-C (Fall 2009) and 10-A (Spring 2010) campaigns. Dosimetry was provided by NSRL physicists, Drs. Adam Rusek, I-Hung Chiang and Michael Sivertz. Mice were shipped back to the University of Iowa Animal Care Facility 3–5 days after irradiation and observed for tumor development. The average age of mice at time of exposure was approximately 6 weeks, with a range of 4.0–9.2 weeks. Mice demonstrating signs of thymic lymphoma (hunching, lethargy) were euthanized. At time of death mice were examined for signs of thymic lymphoma (enlarged thymus, enlarged spleen) and tissues were placed into 10% neutral-buffered formalin. Thymic lymphoma was determined to be the cause of death when Lck-Bax mice exhibited hunched, lethargic behavior and were found at necropsy to have a grossly enlarged thymus, often with spleen/liver involvement.

DHE Oxidation Assessment by Flow Cytometry and MnSOD Activity Determinations

Six- to 10-week-old mice were euthanized and the thymus was excised, minced between glass slides in cold PBS to extract thymocytes, which were then filtered through a cell strainer into a 50 mL conical tube and centrifuged at 300g for 5 min. The supernatant was decanted and thymocytes were resuspended in 1 mL of PBS with 0.5% BSA. The thymocyte suspension was counted with a Beckman Coulter Z1. DHE oxidation was measured on a Becton Dickinson LSR II Flow Cytometer equipped with a violet laser with an excitation of 405 nm to detect superoxide-specific DHE oxidation in isolated thymocytes as previously described (24, 25). MnSOD activity was measured as previously described on whole thymus homogenates prepared on ice in 50 mM potassium phosphate buffer (26). The assay measures MnSOD activity based on the competition between MnSOD and an indicator molecule (nitroblue tetrazolium) that reacts with superoxide generated by xanthine/xanthine oxidase in the presence of 5 mM sodium cyanide to inhibit CuZnSOD activity (26).

Survival Curves and Statistical Analysis

Survival analysis methods were used to estimate and compare the *in vivo* effects of treatment on disease-specific survival. The survival endpoints were time to death due to lymphomagenesis. Mice that were alive at the end of the experiment were treated as censored observations. In addition, mice that died or were euthanized for conditions unrelated to thymic lymphoma (including mice where cause of death could not be determined) during the experiment were treated as censored observations in the analysis. Kaplan-Meier survival plots were constructed to estimate survival curves as a function of time. The log-rank test was used to compare survival between the additional treatment groups. All tests were two-sided and carried out at the 5% level of significance. Analyses were performed with the SAS 9.3 software package (Cary, NC). For DHE and MitoSOX oxidation statistical analysis was carried out with a two-way ANOVA and Tukey's post hoc test.

RESULTS

Gene Dosage Determines Lymphoma-Free Survival Duration in Lck-Bax Mice

The number of Lck-Bax transgenes determines the overall rate of lymphoma-free survival in C57B/6 mice with the Lck-Bax1 (one transgene) and Lck-Bax38&1 (two trans-genes) survival plots shown in Fig. 1A. Lck-Bax-Negative wild-type mice developed only one case of spontaneous thymic lymphoma while the median survival of Lck-Bax1 mice was approximately 70 weeks and thymic lymphoma-genesis was further increased in Lck-Bax38&1 mice demonstrating a median survival of approximately 28 weeks. Gender was

not a significant factor in the development of lymphoma for Bax-Negative or Bax38&1 mice shown in Fig. 1B and D. However, in the Bax1 genotype, female mice demonstrated significantly increased lymphoma free survival compared to male mice when observed to 65 weeks of age (Fig. 1C).

Ten cGy of Low-LET ^{137}Cs Accelerates Lymphomagenesis in Lck-Bax1 Female Mice

Lck-Bax1 mice trended toward accelerated lymphoma-genesis in the combined gender cohort (Fig. 2A) after exposure to either 10 or 100 cGy ^{137}Cs . The greatest trend in accelerated lymphomagenesis was seen after the 10 cGy dose ($P = 0.1861$). When female Lck-Bax1 mice were examined (Fig. 2B), 10 cGy significantly accelerated lymphomagenesis, increasing both the rate of lymphoma-genesis and also the penetrance of thymic lymphomagenesis in female Lck-Bax1 mice. Median lymphoma-free post-exposure survival after 10 cGy ionizing radiation was 32 weeks, while median lymphoma-free survival in unirradiated female Lck-Bax1 mice was over 60 weeks. Interestingly, a higher dose of 100 cGy was less effective than 10 cGy at accelerating the rate and increasing the penetrance of thymic lymphomagenesis in female mice (Fig. 2B). Radiation exposure had no effect on male Lck-Bax1 lymphomagenesis (Fig. 2C).

Low-LET ^{137}Cs Demonstrates a Dose-Independent Gender-Specific Trend Toward Increasing Lymphomagenesis in Lck-Bax38&1 Mice

Lck-Bax38&1 mice exposed to ^{137}Cs radiation, as shown in Fig. 3A, demonstrated a dose-independent trend toward accelerated lymphomagenesis. Median time to death for sham-irradiated, 10 and 100 cGy exposed Lck-Bax38&1 mice was 23, 19.6 and 24.5 weeks, respectively. These results also demonstrated a trend toward female Lck-Bax38&1 mice being susceptible to accelerated lymphomagenesis after a single dose of 10 cGy ^{137}Cs ($P = 0.0761$, Fig. 3B) while both sham and 100 cGy groups demonstrated no gender-effect trend (data not shown).

Lymphomagenesis is not Significantly Altered by a Single Exposure to 10 or 100 cGy of 300 MeV/n Silicon HZE in Lck-Bax38&1 Mice

Irradiation with silicon ions (LET 67 keV/ μm) had no effect on overall lymphoma-free survival in Lck-Bax38&1 mice, as shown in Fig. 4. In addition, gender-specific effects were not noted at any dose level after exposure to silicon ions (data not shown). Median time to death after sham irradiation of Lck-Bax38&1 mice in this cohort was 18.4 weeks, while 10 and 100 cGy groups demonstrated a statistically insignificant increase in median time to death (21.4 and 20.2 weeks, respectively).

Lymphomagenesis is Accelerated in Lck-Bax38&1 Mice after Exposure to a Single Dose of High-LET 1,000 MeV/n Iron Ions

As shown in Fig. 5A, a single dose of 100 cGy iron particle HZE radiation significantly accelerated time to death from lymphoma in Lck-Bax38&1 mice by 5.6 weeks (median survival 16.5 weeks after irradiation, $P = 0.0067$) compared to untreated mice (median survival 22.8 weeks after sham irradiation). Also observed was a statistically significant dose-response relationship (100 cGy vs. 10 cGy, $P = 0.0272$). A trend ($P = 0.0997$) toward increased susceptibility of female Lck-Bax38&1 mice to accelerated lymphomagenesis after 100 cGy iron ions (compared to males) was also noted (Fig. 5B). Female Lck-Bax38&1 mice median survival was reduced to 13.5 weeks while male Lck-Bax38&1 mice median survival was 18.1 weeks.

Loss of Sirtuin 3 Increases Levels of DHE Oxidation and Accelerates the Onset of Lck-Bax Mediated Lymphomagenesis

Thymocytes from four different genetically modified mouse models (WT, Sirt3^{-/-}, Lck-Bax38&1 and Sirt3^{-/-}/Lck-Bax38&1) were examined *ex vivo* using flow cytometry to assess DHE and MitoSOX oxidation levels per cell as markers for superoxide production. Results show in Fig. 6A confirm the previously reported 1.5-fold increased DHE oxidation when WT thymocytes are compared with Lck-Bax38&1 thymocytes (4). Interestingly, the combination of both Sirt3 loss and Bax over expression in Sirt3^{-/-}/Lck-Bax38&1 thymocytes significantly increases DHE and MitoSOX oxidation levels compared to WT, Sirt3^{-/-}, and Lck-Bax38&1 thymocytes (Tukey post hoc *P* values of <0.001, 0.003 and 0.032 for DHE and *P* < 0.001 for MitoSOX when comparing Sirt3^{-/-}/Lck-Bax38&1 to WT, Sirt3^{-/-} and Lck-Bax38&1, respectively). MnSOD activity (Fig. 6C) was significantly decreased (*P* < 0.05, Student's *t* test) in Sirt3^{-/-}/Lck-Bax38&1 compared to Lck-Bax38&1.

The Kaplan-Meier survival plot in Fig. 6D demonstrates that the loss of Sirt3 in Bax38&1 mice (Sirt3^{-/-}/Bax38&1) significantly (*P* < 0.0001) accelerated thymic lymphomagenesis when compared to Bax38&1 mice with WT Sirt3 expression (Bax38&1). Median survival decreased by approximately 7 weeks in Sirt3^{-/-}/Lck-Bax38&1 mice compared to Bax38&1 while maximum survival decreased by nearly half from 52 weeks in Lck-Bax38&1 to only 28 weeks in Sirt3^{-/-}/Lck-Bax38&1 mice.

DISCUSSION

The current *in vivo* study of mitochondrial dysfunction and radiation response highlights LET and gender as key factors altering responses. Overall these studies demonstrate the susceptibility of female mice to both low-LET ¹³⁷Cs rays and high-LET 1,000 MeV/n iron particles in both the Lck-Bax1 and Lck-Bax38&1 models. Most notably, 10 cGy of ¹³⁷Cs significantly accelerated the rate and increased the prevalence of thymic lymphoma specifically in female Lck-Bax1 mice; where the effects of gender, metabolism and aging have a longer time to manifest prior to the onset of lymphomagenesis. This effect is especially significant as female Lck-Bax1 mice survived longer than male Lck-Bax1 mice in the absence of radiation exposure.

Experiments performed with Lck-Bax38&1 mice reveal a significant difference between two different HZE particles (iron and silicon) differing in LET, fluence and biological effectiveness at accelerating lymphomagenesis. Iron ions (1,000 MeV/n, 150 keV/μm) significantly accelerated lymphomagenesis in Lck-Bax38&1 mice after a single dose of 100 cGy, while silicon ions (300 MeV/n, 67 keV/μm) had no effect at either 10 or 100 cGy. Iron ion exposure led to a trend in acceleration of lymphomagenesis in female Lck-Bax38&1 mice compared to males, while both genders of Lck-Bax38&1 mice were unaffected by silicon ion exposure.

The lack of an effect with silicon ions at isodoses compared to iron ions in the current study was surprising. It has been demonstrated with cells in culture that both iron and silicon ions at numerous energies tested exhibit RBEs >1 and are effective at inducing complex biological damage that is not easily repaired (27, 28). One factor for consideration regarding the current finding of iron ions having greater effect than silicon ions is fluence, where 2.5 times as many 67 keV/μm (300 MeV/n) silicon ions traverse the animal at a given dose level compared to the more densely ionizing 150 keV/μm (1,000 MeV/n) iron ions where fewer particle tracks are required to achieve the desired energy deposition (dose). Therefore, it is possible that an LET threshold exists for acceleration of lymphomagenesis in Lck-Bax38&1 mice due to the physical characteristics of the high-LET beams being inversely proportional to fluence. Previously it has been demonstrated on a fluence basis that 1,000 MeV/n iron

ions are more effective at clonogenic cell killing compared to 490 MeV/n silicon, supporting the concept of maximal RBE at LETs of approximately 100–150 keV/μm (27). A further distinction between 1,000 MeV/n iron and 300 MeV/n silicon ions is delta-ray generation. Iron ions used in this study generate a greater field of delta rays (low-LET photon irradiation) emanating out from the particle track as they traverse a biological matrix compared to silicon ions. The importance of delta-ray generation to high-LET radiation response is significant, and thus a factor to consider when interpreting the results from the current iron and silicon particle experimental groups (29). Based on the results from the current study of high-LET GCR HZE particles, only iron caused a dose-dependent acceleration of lymphomagenesis in Lck-Bax38&1 mice, while silicon HZE particle exposure at up to 100 cGy did not alter lymphoma-free survival. This suggests that thymic cell transformation potency may differ significantly between different types of HZE-particle exposures.

Gender-based differences in sensitivity to acceleration of lymphomagenesis after ionizing radiation exposure were a surprising finding of the current study. Lck-Bax1 female mice demonstrated acute susceptibility to low dose, low-LET ionizing radiation, as evidenced by not only an increased rate of lymphomagenesis, but also a striking increase in the overall penetrance of lymphomagenesis. Not a single irradiated female Lck-Bax1 mouse survived past one year of age compared to sham-irradiated females where only 20% succumbed to thymic lymphoma in the first year. Interestingly, male Lck-Bax1 lymphomagenesis was unchanged after irradiation with either 10 or 100 cGy of ^{137}Cs . Gender effects were also noted in low-LET and high-LET Lck-Bax38&1 cohorts where 100 cGy of 1,000 MeV/n iron-HZE ions and 10 cGy of ^{137}Cs rays demonstrated trends toward increased sensitivity in female mice relative to male mice (*P* values of 0.0997 and 0.0761, respectively). As our studies were originally designed with sufficient power to detect differences in responses to heavy ions and ^{137}Cs when male and female groups were pooled, the inability to detect a statistically significant difference in radiation sensitivity comparing male versus female Lck-Bax38&1 mice is not unexpected given the overall acceleration of lymphomagenesis in Lck-Bax mice caused by the expression of two transgenes.

The biological potency of high-LET iron ion irradiation over cesium photons at equivalent doses is well characterized *in vitro* (27, 28). However, a recent investigation of iron ion-induced leukemia in mice found an RBE of 1, rather than previously calculated RBEs of 3–4 for 1,000 MeV/n iron ions compared to ^{137}Cs rays (30). The current study, examining lymphomagenesis in the C57B/6 background, demonstrates that a 100 cGy dose of iron-HZE ions has a higher biological effectiveness at accelerating lymphomagenesis in Lck-Bax38&1 mice compared to 100 cGy of low-LET ^{137}Cs rays. Furthermore, the effect of iron-HZE ions on lymphomagenesis was dose dependent, while the effects of ^{137}Cs rays demonstrated an apparent inverse dose dependency in Lck-Bax38&1 over expressing animals (10 cGy of ^{137}Cs radiation demonstrated a significantly greater acceleration of lymphomagenesis in female Lck-Bax1 mice relative to 100 cGy). These results support the concept that there is a dose response relationship for high-LET GCR in this lymphomagenesis model system, that may be relevant for informing risk assessment on extended Mars missions of 1,000 days where it is estimated a cumulative dose of 40 cGy is anticipated (31). Understanding which cell types are most susceptible to HZE particle radiation is a critical task in ascertaining an accurate cancer induction risk for these highly energetic and damaging ions.

The underlying etiology for female susceptibility to ionizing radiation has many suspected mechanisms, but overall is poorly understood. Exposure to <1 Gy of ionizing radiation was previously thought to have no connection to lymphomagenesis, but recent data suggest that lymphomagenesis is a very late effect in exposed human populations (10, 12, 13).

Unfortunately, the epidemiological study linking ionizing radiation exposure to increased risk of lymphoma excluded females to focus solely on occupationally exposed men in the U.S. and Japanese men from the Lifespan Study cohort. Nevertheless, a dose-response association between radiation exposure and lymphoma mortality with an extended latency period was demonstrated, underscoring the importance of studying the role of ionizing radiation response and how mitochondrial dysfunction impacts lymphomagenesis.

The critical role of MnSOD in this model was demonstrated in Lck-Bax38&1 mice, where deletion of one SOD2 allele significantly accelerated thymic lymphomagenesis (4). Loss of MnSOD activity and increased superoxide production has been proposed as a crucial event in cellular neoplastic transformation, and molecular biology techniques employed to increase MnSOD activity and lower superoxide levels have the effect of slowing cancer cell growth in several model systems (32–36). To mechanistically test the role of mitochondrial oxidative metabolism in mediating the promotion phase of carcinogenesis in Lck-Bax mediated thymic lymphomagenesis, Sirt3 knockout mice were crossed with thymocyte specific Lck-Bax overexpressing mice. Sirtuin 3 is a mitochondrial protein deacetylase and responsible for controlling many aspects of superoxide metabolism (21, 22, 37). If superoxide is in fact driving Bax-mediated thymic lymphomagenesis, the loss of Sirt3 would be predicted to further increase mitochondrial dysfunction, lead to increased superoxide and other ROS generation, and result in the acceleration of thymic lymphomagenesis. Figure 6 demonstrates that loss of Sirt3 markedly increases DHE oxidation, which is a general marker of superoxide levels as well as MitoSOX oxidation, which is targeted to the mitochondria by virtue of a cationic triphenylphosphonium moiety (Fig. 6A and B) (38). In addition, loss of Sirt3 led to decreased MnSOD activity in the premalignant thymus of Sirt3^{-/-}/Bax38&1 mice and a significant acceleration of thymic lymphomagenesis in Lck-Bax38&1 mice (Fig. 6C and D). These results support the hypothesis that mitochondrial superoxide is promoting lymphomagenesis in the Lck-Bax model and that Sirt3 is regulating mitochondrial superoxide production to counteract the carcinogenic stimulus of excess superoxide production from Bax overexpression.

Sirtuin 3's role during carcinogenesis is currently somewhat controversial (39–41). Sirtuin 3 was reported to be upregulated in one study of human oral squamous cell carcinoma, while knockdown of Sirt3 sensitized these cancer cells to therapy (42). In contrast to head and neck tumors, most other studies have reported Sirt3 expression is decreased in human cancers as they progress toward malignancy (21, 43). Sirtuin 3's mechanistic role as a tumor suppressor has been demonstrated through stabilization of p53, negative regulation of metabolic changes favoring glycolysis, detoxification of superoxide anion by deacetylation of MnSOD increasing activity, and through apoptotic pathways (22, 41, 44–47). In addition, Sirt3^{-/-} mouse embryonic fibroblasts (MEFs) were transformed by only a single oncogene, whereas Sirt3^{+/+} MEFs required two oncogenes for transformation (21). The current data clearly demonstrate that loss of Sirt3 accelerates thymic lymphomagenesis in Lck-Bax38&1 mice, consistent with previous studies suggesting Sirt3 was a mitochondrial localized tumor suppressor (21, 22, 39, 41, 43, 45–48).

Defects in mitochondrial metabolism and excess superoxide generation are increasingly recognized as contributing to disease processes, especially carcinogenesis (5, 34, 48–50). The current study of radiation-induced acceleration of lymphomagenesis in mice with altered mitochondrial metabolism demonstrates that the most densely ionizing radiation was most effective overall at high doses. However, 10 cGy of sparsely ionizing ¹³⁷Cs rays markedly accelerated lymphomagenesis in female Lck-Bax1 mice. In addition, the ineffectiveness of silicon ions at accelerating lymphomagenesis in either male or female Lck-Bax38&1 mice could suggest that the target organ for silicon ions may not be the thymus.

In conclusion our findings support the hypothesis that genetic manipulations altering superoxide and mitochondrial oxidative metabolism significantly alter *in vivo* radiation response to low doses of both high-LET iron ions and low-LET ^{137}Cs photons. Female susceptibility to the detrimental effects of ionizing radiation, shown in this study as accelerated lymphomagenesis, suggests that mitochondrial oxidant production may interact with gender and aging to sensitize a subpopulation of individuals to the damaging effects of radiation exposure from low doses. In addition, these studies also present a novel role for Sirt3 in controlling thymic cell superoxide levels and cancer induction in the Lck-Bax model of lymphomagenesis.

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FIG. 1.

Lymphoma-free survival is dependent upon the number of Lck-Bax transgenes present in C57B/6 mice. Panel A: Lck-Bax38&1, Lck-Bax1 and WT lymphoma-free survival using Kaplan Meier methodology demonstrates that the number of Lck-Bax transgenes (2 in Lck-Bax38&1, 1 in Lck-Bax1 and 0 in WT mice) controls the rate and penetrance of thymic lymphomagenesis in C57B/6 mice. Panels B–D: Male and female WT and Lck-Bax38&1 mice develop lymphoma at the same rate, however Lck-Bax1 female mice survive significantly longer than male Lck-Bax1 mice.

FIG. 2. Low-LET ^{137}Cs 10 cGy exposure accelerates lymphoma-genesis in female Lck-Bax1 mice. Panel A: A mixed gender cohort of Lck-Bax1 mice demonstrates a dose independent trend for accelerated lymphomagenesis after 10 and 100 cGy of low-LET ^{137}Cs exposure. Panel B: Female Lck-Bax1 mice demonstrate significantly accelerated lymphomagenesis in response to 10 cGy of ^{137}Cs . Panel C: Lymphomagenesis is unchanged in irradiated male Lck-Bax1 mice.

FIG. 3.

Lck-Bax38&1 mice exposed to a single dose of low-LET ^{137}Cs demonstrate an inverse dose response trend toward accelerated lymphomagenesis. Panel A: Male and female Lck-Bax38&1 mice were exposed to 0, 10 and 100 cGy of low-LET ^{137}Cs and monitored for lymphoma-free survival. A trend toward accelerated lymphomagenesis was noted in 10 cGy irradiated Lck-Bax38&1 mice that was not apparent in 100 cGy irradiated mice. Panel B: 10 cGy ^{137}Cs irradiation demonstrates a strong trend ($P = 0.0761$) toward acceleration of lymphomagenesis specifically in female Lck-Bax38&1 mice compared to males.

FIG. 4. Lymphomagenesis is unchanged after a single 10 or 100 cGy dose of 300 MeV/n silicon ions in Lck-Bax38&1 mice. Lck-Bax38&1 mice were exposed to 0, 10 or 100 cGy of 300 MeV/n silicon ions (~67 keV/μm) and lymphomagenesis was unchanged in all groups. Gender-specific effects were not seen at 0, 10 or 100 cGy (data not shown).

FIG. 5.

Lymphomagenesis is accelerated in a dose-dependent manner in Lck-Bax38&1 mice after exposure to high-LET 1,000 MeV/n iron ions. Panel A: Lck-Bax38&1 mice were administered doses of 0, 10 or 100 cGy of 1,000 MeV/n iron ions (~150 keV/μm) and monitored for lymphoma-free survival. One hundred cGy of iron ions significantly accelerated lymphomagenesis, while 10 cGy demonstrated only a slight trend toward accelerating lymphomagenesis. Panel B: Female Lck-Bax38&1 mice exposed to 100 cGy of 1,000 MeV/n iron ions demonstrated a trend toward heightened sensitivity to acceleration of lymphomagenesis compared to 100 cGy exposed male Lck-Bax38&1 mice ($P=0.0997$). Lymphomagenesis in 0 and 10 cGy irradiated Lck-Bax38&1 mice was not influenced by gender (data not shown).

FIG. 6.

Loss of Sirt3 increases superoxide levels in Lck-Bax thymocytes and accelerates onset of thymic lymphoma. Panels A and B: Thymocytes isolated from WT, Sirt3^{-/-}, Lck-Bax38&1 and Sirt3^{-/-}/Lck-Bax38&1 mice were labeled with DHE (panel A) or MitoSOX (panel B) and the mean fluorescence intensity (MFI) was calculated by flow cytometry. DHE and MitoSOX oxidation trended toward a slight increase in Sirt3^{-/-} and Lck-Bax38&1 thymocytes compared to WT mice. The combination of Sirt3 loss and Bax overexpression in Sirt3^{-/-}/Lck-Bax38&1 thymocytes markedly enhanced both DHE and MitoSOX oxidation to a greater extent than either genetic manipulation alone. **P* < 0.05. Panel C: MnSOD activity was measured in whole thymus from WT, Sirt3^{-/-}, Lck-Bax38&1 and Sirt3^{-/-}/Lck-Bax38&1 mice and found to be significantly lower in Sirt3^{-/-}/Lck-Bax38&1 mice. **P* < 0.05, Student's *t* test (panel D) Sirt3^{-/-}/Lck-Bax38&1 mice develop thymic lymphoma at an accelerated rate compared to Sirtuin 3 WT Lck-Bax38&1 mice. WT and Sirt3^{-/-} mice only developed one case of thymic lymphoma in the first year of life (Fig. 1 and data not shown).