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Devastation of Adult Stem Cell Pools by Irradiation Precedes Collapse of Trabecular Bone Quality and Quantity

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ABSTRACT

Stem cell depletion and compromised bone marrow resulting from radiation exposure fosters long-term deterioration of numerous physiologic systems, with the degradation of the skeletal system ultimately increasing the risk of fractures. To study the interrelationship of damaged bone marrow cell populations with trabecular microarchitecture, 8- and 16-week-old C57BL/6 male mice were sublethally irradiated with 5 Gy of ^{137}Cs γ-rays, and adult stem cells residing in the bone marrow, as well as bone quantity and quality, were evaluated in the proximal tibia after 2 days, 10 days, and 8 weeks compared with age-matched controls. Total extracted bone marrow cells in the irradiated 8-week, young adult mice, including the hematopoietic cell niches, collapsed by 65% $\pm$ 11% after 2 days, remaining at those levels through 10 days, only recovering to age-matched control levels by 8 weeks. As early as 10 days, double-labeled surface was undetectable in the irradiated group, paralleled by a 41% $\pm$ 12% and 33% $\pm$ 4% decline in bone volume fraction (BV/TV) and trabecular number (Tb.N), respectively, and a 50% $\pm$ 10% increase in trabecular separation (Tb.Sp) compared with the age-matched controls, a compromised structure that persisted to 8 weeks postirradiation. Although the overall collapse of the bone marrow population and devastation of bone quality was similar between the "young adult" and "mature" mice, the impact of irradiation—and the speed of recovery—on specific hematopoietic subpopulations was dependent on age, with the older animals slower to restore key progenitor populations. These data indicate that, independent of animal age, complications arising from irradiation extend beyond the collapse of the stem cell population and extend toward damage to key organ systems. It is reasonable to presume that accelerating the recovery of these stem cell pools will enable the prompt repair of the skeletal system and ultimately reduce the susceptibility to fractures.

KEY WORDS: HEMATOPOIETIC STEM CELLS; MESENCHYMAL STEM CELLS; RADIATION; BONE MARROW SUPPRESSION; NICHE

Introduction

Many bone marrow disorders, including chronic myelogenous leukemia, acute myelogenous leukemia, multiple myeloma, and aplastic anemia, are associated with bone loss.[1–3] Oftentimes, radiation and chemotherapy treatment contribute to, if not specifically target, bone marrow suppression and thus inherently contribute to the progressive loss of bone and the increase of fracture risk.[4] Radiation exposure is often associated with medical therapeutics and diagnostics, but astronauts in space and those adjacent to nuclear calamities may also suffer from dangerous levels of accidental exposure. Ultimately, it is important to preserve bone strength, a goal that is most typically approached via antiresorptive treatments of the bone tissue, targeting the resident bone cell population.[5,6] Given that the bone marrow space serves as the primordial pool to both bone-forming osteoblasts and bone-resorbing osteoclasts, it is essential to nurture the recovery of this progenitor pool as a potential means of, among other things, restoring bone quantity and quality. This bone marrow stem cell niche includes the mesenchymal stem cell (MSC) population, responsible for osteogenic, adipogenic, and chondrogenic differentiation, and the hematopoietic stem cell (HSC) population, which provides continuous renewal of blood cell lineages and the foundation of the immune system.[7,8] Indeed, it would appear that the restoration of these two stem cell pools after marrow insult represents a first critical step to the recovery of many physiologic systems.[9]

Hematopoietic cells are essential to controlling the immune system, and detailed methods now exist to allow for isolation of the stem cell population to determine the cells responsible for specific processes.[10,11] HSCs (SP-LSK) are capable of self-renewal

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and are also able to differentiate into $Lin^{-}Sca\text{-}1^{+}C\text{-}kit^{+}$ progenitor cells (LSK), critical to maintenance of hematopoiesis and proper immune function because they give rise to myeloids and lymphocytes. However, insults such as irradiation can hinder hematopoiesis and disrupt proper HSC self-renewal and represent a major challenge for long-term bone retention and repair.[12–14] The impact of irradiation on the viability of the MSC population is a controversial issue, but in vitro studies indicate that radiation can hinder the capacity of MSCs to proliferate and differentiate.[15,16] Because MSCs and HSCs share the same niche within the bone marrow and the two populations often interact to control their stem cell function,[17,18] the ability to protect or reestablish the bone marrow phenotypic populations could potentially accelerate the repair and restoration of the bone's structure and strength.

In the work presented here, we expose young adult (8-week) and skeletally mature (16-week) adult mice to total body sublethal irradiation in an effort to define the degree to which specific components of the bone marrow population are affected and the degree to which these changes correlate with changes in bone quality. We hypothesize that the irradiation exposure will rapidly impact the bone marrow stem cell pool, devastation that will precede destruction of bone architecture.

Materials and Methods

Animals

All studies were reviewed and approved by the State University of New York at Stony Brook's Institutional Animal Care and Use Committee. Six-week-old (young adult) and 14-week-old (mature adult) male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA) were acclimated for 2 weeks before the start of the study, housed individually, and were given food and water ad libitum. For each age group, mice were randomly divided into two groups, which included irradiated (Irrad) and age-matched sham controls (control). Food consumption was monitored and animals were weighed weekly. In the young Irrad group, 8 mice were euthanized at 2 days, 10 days, and 8 weeks, whereas the control group had 7 mice at 2 days and 8 mice at 10 days and 8 weeks. The mature adult Irrad and control mice had 10 mice in each group euthanized at 2 days, 10 days, and 8 weeks (Fig. 1).

Radiation-induced bone marrow damage

Eight-week-old and 16-week-old mice in the Irrad groups were placed, unconstrained, into a ^{137}Cs γ-ray irradiation chamber, four mice at a time, and exposed to 5 Gy of total body sublethal irradiation at 0.60 Gy/minute for 8.4 minutes without shielding. Control mice were placed in the inactive irradiator to serve as sham controls.

Assessing hematopoietic stem cell lineages in the bone marrow using flow cytometry

At euthanization, bone marrow was flushed with supplemented Dulbecco's Modified Essential Medium (Gibco, Carlsbad, CA, USA) containing 2% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 10 mM Hepes buffer (Gibco), and 1% penicillin/streptomycin from the right tibia and femur, mixed to obtain a single-cell suspension, and filtered through a 40-μm filter (BD Biosciences, San Diego, CA, USA). The red blood cells were lysed using 1X PharmLyse (BD Biosciences), and the remaining cells were resuspended in the supplemented medium. The number of cells present was quantified using an automatic cell counter, Scepter (Millipore, Billerica, MA, USA). To perform flow cytometry analysis (FACSCalibur and FACSAria; BD, San Jose, CA, USA), 2×10^6 cells were stained for leukocytes using APC-conjugated CD45/B220, CD11b/Mac-1, Ly-6G/Ly-6C, and PE-conjugated CD45/B220, CD4, and CD8 (BD Biosciences).[10] LSK cells in the bone marrow were quantified with antibodies for Lin^{-}, $Sca\text{-}1^{+}$, and $C\text{-}kit^{+}$, on 2×10^6 cells. SP-LSK cells were quantified using Vybrant DyeCycleViolet (Invitrogen) according to the manufacturer's protocol. For leukocyte analysis in the peripheral blood, 100 μl of whole blood obtained via cardiac puncture was lysed using 1X PharmLyse and the same markers were used for leukocyte quantification as used for the bone marrow quantification.

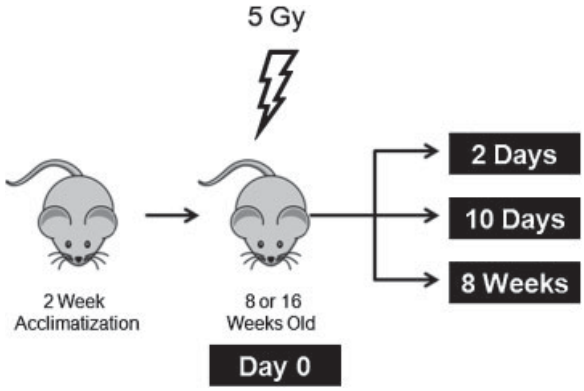

Fig. 1. Experimental schematic of mice that were acclimated for 2 weeks and irradiated with a 5 Gy sublethal dose of γ-irradiation at 8 or 16 weeks of age. The mice were then euthanized at 2 days, 10 days, or 8 weeks after irradiation.

Apoptosis quantification

The TUNEL TACS 2 TdT-Blue Label In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA) was used to qualitatively assess the apoptotic cells present in the bone marrow according to the manufacturer's protocol.

Assessing bone microarchitectural recovery using micro-computed tomography (μCT)

The left tibias were extracted and stored at −20°C in 70% ethanol. The proximal tibia was scanned with an ex vivo μCT 40 (Scanco Medical, Bassersdorf, Switzerland) using 12-μm isotropic voxels. Using a well-established script,[19] an 840-μm region was evaluated 300 μm distal to the growth plate to determine the bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), and connectivity density (Conn.D).

Quantifying osteoclast activity

The MouseTRAP Assay Kit (Immunodiagnostics Systems, Scottsdale, AZ, USA) was used to detect the amount of

osteoclast-derived tartrate-resistant acid phosphatase form 5b (TRAP) in the mouse serum at 2 days after irradiation and was followed according to the manufacturer's protocol.

Dynamic histomorphometry

Mice scheduled for euthanization at 8 weeks postirradiation, as well as their age-matched controls, were labeled with 10 mg/kg of calcein diluted in PBS at 4 weeks, followed by a second ip injection 6 days later. Those scheduled for sacrifice at 10 days were given the same labels but at 2 days and 8 days after irradiation. Two-day animals were not labeled. The tibias first used for μCT scanning were embedded in polymethylmethacrylate using the following procedure: Bones were dehydrated in 70%, 95%, and 100% isopropyl alcohol, cleared in petroleum ether, and underwent three infiltration steps. The first infiltration solution contained 85% methyl methacrylate and 15% n-butyl phthalate; the second was 85% methyl methacrylate, 15% n-butyl phthalate, and 1% w/v benzoyl peroxide; and the third infiltration was 85% methyl methacrylate, 15% n-butyl phthalate, and 2% w/v benzoyl peroxide. The samples were embedded in 20-mL scintillation vials with an 85% methyl methacrylate, 15% n-butyl phthalate, and 2% w/v benzyl peroxide solution, and placed in a 37°C water bath for 3 days to solidify. The tibias were cut in 6-μm coronal sections using a microtome (Leica, Bannockburn, IL, USA). Osteomeasure software (OsteoMetrics, Decatur, GA, USA), which performs standard histomorphometry analysis, was used to quantify single-labeled surface, double-labeled surface, mineral apposition rate (MAR), and bone formation rate per bone surface (BFR/BS) in the trabecular bone distal to the growth plate.[20]

Statistical analysis

Numerical data are presented as mean ± standard deviation. Statistical analysis between Irrad and age-matched controls was performed in SPSS using an independent samples *t* test. Statistical analysis between the young and mature mice compared with their age-matched controls was performed in SPSS using a one-way ANOVA followed by a Tukey post hoc test. A p value <0.05 was considered significant.

Results

Difference between young and mature control mice

The young (8-week) and mature (16-week) mice were 22.3 ± 1.6 g and 28.7 ± 1.6 g at the commencement of the study, indicating the older animals to be 28.7% heavier than the younger mice. However, as indicated by the rapid increase in weight of the young mice, these animals were rapidly growing throughout the study, whereas the mature adult mice remained close to the same weight over the 8-week experimental period. Despite the differences in age and weight, the leukocyte phenotypic populations did not vary between the young and mature adult control mice throughout the study, with the exception of the T-cell phenotype, which showed a 50% decline from the 8-week-old to the 24-week-old mice (measured from marrow harvested at the end of the 8-week period in the older mice). More specifically, the T-cell population comprised 1.34% ± 0.05%, 1.20% ± 0.31%, and 0.71% ± 0.16% of the bone marrow in 8-week, 16-week, and 24-week mice, respectively ($p < 0.05$).

Radiation-induced weight loss

The immediate impact of irradiation was established by calculating changes in whole body and isolated organ mass. Young and mature adult mice euthanized 2 days postirradiation experienced a −0.59 ± 0.60 g (−2.6%) and −1.30 ± 0.64 g (−4.5%) drop in whole body weight compared with baseline, whereas the controls increased 0.47 ± 0.30 g (3%) and 0.11 ± 0.58 g (0.4%), respectively. Fig. 2 shows the weight of the mice over 8 weeks as a proportion of their individual weight at the commencement of the study. By 2 weeks after irradiation, no significant differences were evident in the proportional weight of mature Irrad mice compared with their age-matched controls, whereas the proportional weight of young Irrad mice remained lower than the young controls up to 5 weeks postexposure. At no point through the study were there any significant differences in food consumption between the Irrad mice and their age-matched controls.

By 2 days, and as compared with their age-matched controls, weights of the thymus and spleen of the Irrad mice had declined

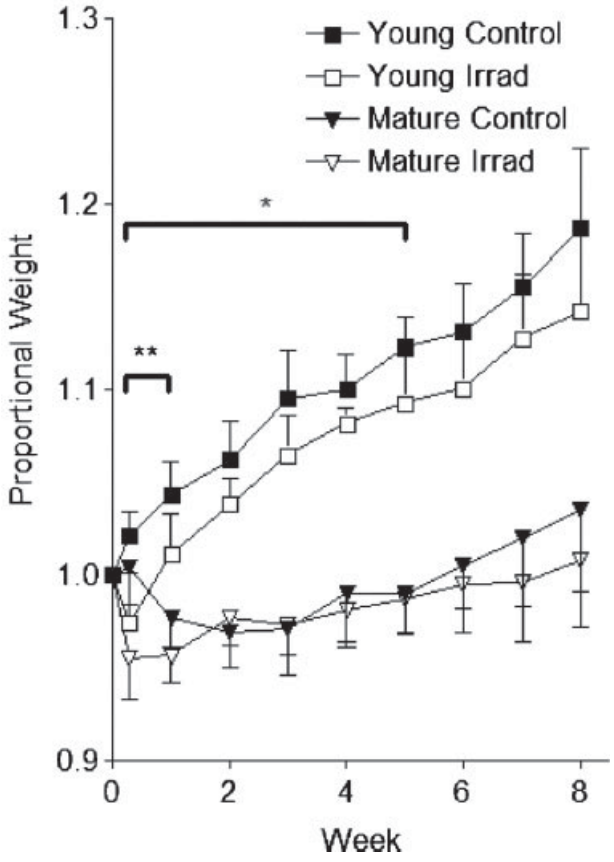

Fig. 2. Proportional change in weight over the 8 weeks after irradiation compared with the commencement of the study. The young and mature Irrad mice experienced a drop in mass at 2 days after irradiation, and the young mice continued to gain weight throughout the study. $^{*}p < 0.05$ when young Irrad are compared with young controls; $^{**}p < 0.05$ when mature Irrad are compared with mature controls.

by $-68\% \pm 14\%$ and $-58\% \pm 4\%$ in the young mice ($p < 0.05$), and $-69\% \pm 8\%$ and $-58\% \pm 3\%$ in the mature mice ($p < 0.05$). Already by 10 days, the irradiated mice showed signs of recovery judging by the increased weights of their thymuses (Table 1). Although recovery of some organ systems was evident, spleens in all Irrad mice were less than half the weight of age-matched controls at 10 days ($p < 0.05$). By 8 weeks, there was still a significant decline in the spleen weight of the mature Irrad mice compared with the age-matched control ($p < 0.05$), but no significant differences in the weights of the thymus and spleen were evident between young control and the young Irrad mice, nor were any differences in kidney and liver weights seen between the control and Irrad throughout the study regardless of animal age.

Irradiation causes rapid depletion of bone marrow

By 2 days, the irradiation had decreased the total number of cells in the bone marrow of young and mature adult mice by $-65\% \pm 11\%$ and $-63\% \pm 18\%$, respectively (Fig. 3), a collapse that showed no evidence of recovery at 10 days. By 8 weeks, however, total cells in the bone marrow of the young Irrad had returned to levels that were not statistically different from their age-matched controls, whereas the mature adult Irrad mice had recovered 88% of the cells compared with age-matched controls, still significantly different from controls ($p < 0.05$) but certainly toward recovery. Although the total number of cells in the bone marrow at 8 weeks in the young Irrad mice had returned to those levels measured in age-matched controls, they still showed evidence of more apoptotic cells with $1.69\% \pm 0.93\%$ of all nucleated cells being apoptotic in the Irrad mice, threefold greater than the $0.63\% \pm 0.18\%$ of apoptotic cells in the age-matched controls ($p < 0.05$, Fig. 4).

Leukocyte phenotypes depleted at altered rates after irradiation

Two days after irradiation, severe injury to the bone marrow was also evident by examining the phenotypic shifts of those bone marrow cells that survived the overall collapse of cells. As determined through FACs analysis, the number of B-cells and T-cells declined by $-97\% \pm 3\%$ and $-92\% \pm 1\%$, respectively, in the young Irrad group compared with its age-matched control ($p < 0.05$; Fig. 5), a collapse similar to that measured in the older irradiated animals at 2 days. By 10 days, the T-cell phenotype showed a slight recovery compared with 2 days in the young Irrad mice but was still $-84\% \pm 3\%$ lower than the age-matched controls ($p < 0.05$). This shift was paralleled by the increase

Table 1. Mice Tissue Weights in the Thymus, Liver, Kidney, and Spleen at 2 Days, 10 Days, and 8 Weeks After Irradiation Presented as Mean ± SD

		Thymus (g)	Liver (g)	Kidney (g)	Spleen (g)
2 Days	Young control	0.044 ± 0.016	1.234 ± 0.101	0.135 ± 0.013	0.059 ± 0.007
	Young Irrad	0.014 ± 0.006	1.215 ± 0.079	0.144 ± 0.015	0.025 ± 0.002
	% Difference	-68.0 ± 13.9	-1.5 ± 6.4	6.6 ± 11.4	-57.8 ± 4.0
	p value	0.000	0.691	0.248	0.000
	Mature control	0.036 ± 0.007	1.386 ± 0.128	0.201 ± 0.022	0.079 ± 0.009
	Mature Irrad	0.011 ± 0.003	1.274 ± 0.098	0.204 ± 0.028	0.033 ± 0.003
	% Difference	-68.6 ± 8.1	-8.1 ± 7.1	1.4 ± 14.1	-58.1 ± 3.3
	p value	0.000	0.040	0.804	0.000
10 Days	Young control	0.038 ± 0.004	1.072 ± 0.100	0.140 ± 0.022	0.068 ± 0.006
	Young Irrad	0.034 ± 0.007	1.027 ± 0.086	0.138 ± 0.014	0.031 ± 0.003
	% Difference	-9.1 ± 18.4	-4.2 ± 8.0	-1.1 ± 10.0	-54.0 ± 4.9
	p value	0.248	0.350	0.866	0.000
	Mature control	0.037 ± 0.005	1.352 ± 0.145	0.183 ± 0.022	0.079 ± 0.018
	Mature Irrad	0.030 ± 0.003	1.274 ± 0.081	0.168 ± 0.011	0.036 ± 0.004
	% Difference	-19.8 ± 7.7	-5.7 ± 6.0	-7.9 ± 5.8	-54.2 ± 4.9
	p value	0.000	0.157	0.073	0.000
8 Weeks	Young control	0.039 ± 0.007	1.311 ± 0.074	0.175 ± 0.018	0.065 ± 0.006
	Young Irrad	0.043 ± 0.004	1.340 ± 0.185	0.165 ± 0.013	0.062 ± 0.008
	% Difference	9.7 ± 10.5	2.2 ± 14.1	-5.7 ± 7.3	-5.6 ± 12.3
	p value	0.246	0.691	0.243	0.320
	Mature control	0.041 ± 0.006	1.438 ± 0.123	0.208 ± 0.022	0.074 ± 0.011
	Mature Irrad	0.041 ± 0.009	1.406 ± 0.108	0.192 ± 0.025	0.065 ± 0.006
	% Difference	0.4 ± 21.9	-2.3 ± 7.5	-7.9 ± 12.0	-12.4 ± 7.8
	p value	0.962	0.553	0.144	0.038

Note: After irradiation, the weights of the thymuses dropped more than 68% and spleens by 58% compared with the age-matched controls and remained lower even at 10 days. By 8 weeks, the weights of the thymuses in all irradiated mice returned to control levels as well as the spleen in the young adult mice. Although the mature Irrad mice showed some recovery of their spleen weights, the spleen weights remained significantly lower than the mature controls.

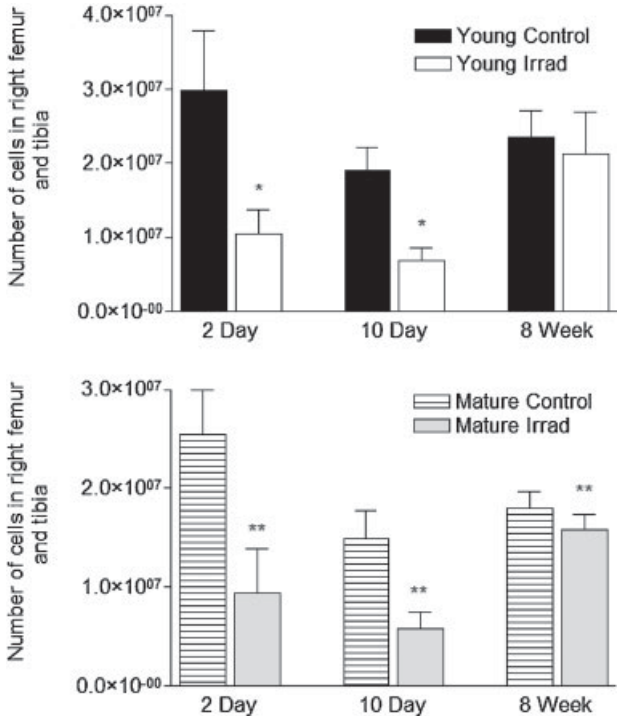

Fig. 3. Bone marrow cellular quantification combined from the right femur and tibia in the control and Irrad mice 2 days, 10 days, and 8 weeks after irradiation. The number of cells in the bone marrow were drastically depleted at 2 days, which continued until 10 days. By 8 weeks, the number of cells in the young Irrad was no longer different from young control, but the mature Irrad still had significantly fewer cells. $^{*}p < 0.05$ when compared with young control; $^{**}p < 0.05$ when compared with mature control.

in thymus mass by 10 days, as discussed above. In contrast, the T-cell population in the mature adult mice was $-80\% \pm 8\%$ lower at 2 days but fell even further at 10 days to $-89\% \pm 4\%$ below the age-matched control. Early stages of bone marrow recovery was also evident in the B-cells in the young Irrad group, which at 10 days was $-64\% \pm 13\%$ lower than the age-matched control, a 33% improvement relative to 2 days. However, the mature mice had not realized the same rate of recovery in the bone marrow, as their B-cells were $-79\% \pm 8\%$ depleted at 10 days, showing

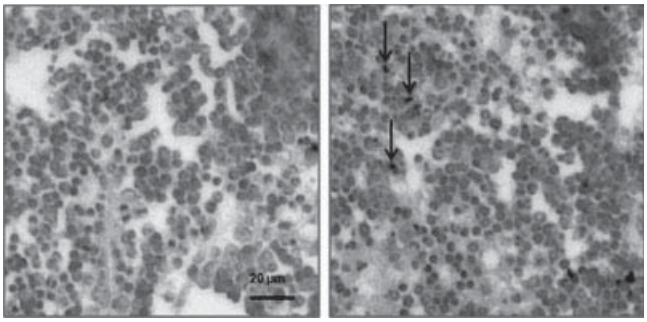

Fig. 4. Apoptotic cells at 8 weeks in the bone marrow of the young control (left) and young Irrad (right) mice stained using the TUNEL labeling kit. Arrows point to a few representative apoptotic cells. Increased numbers of apoptotic cells in the young Irrad mice indicates that the bone marrow remains compromised at 8 weeks even though the total number of cells returned to control levels.

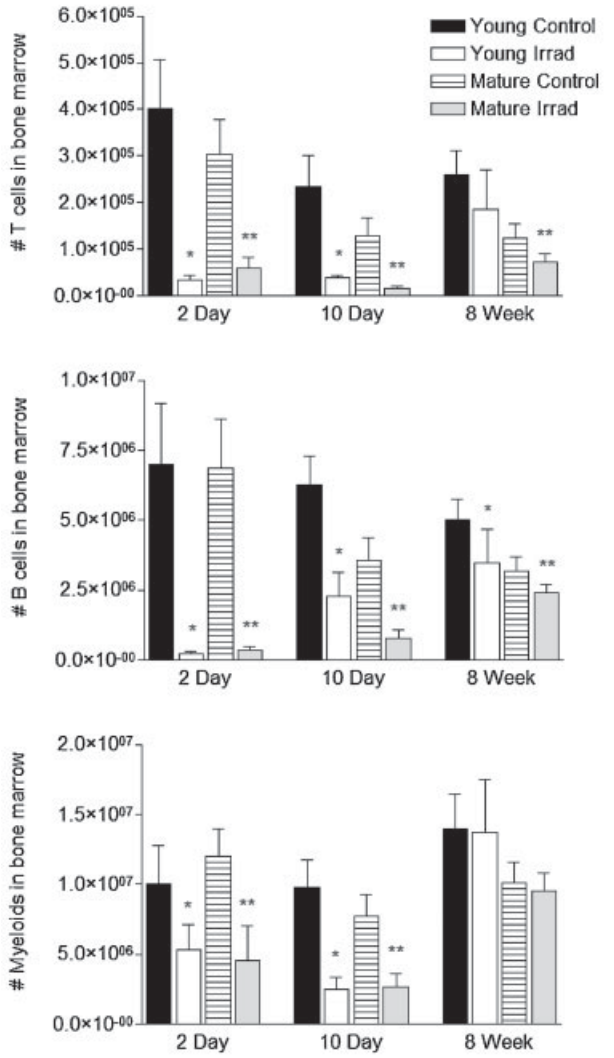

Fig. 5. Phenotypic makeup of the leukocytes from the bone marrow of the right tibia and femur 2 days, 10 days, and 8 weeks after irradiation. A decline in leukocytes was seen at 2 days and persisted until 8 weeks for lymphoids even though the myeloids showed a full recovery at 8 weeks. $^{*}p < 0.05$ when compared with young control; $^{**}p < 0.05$ when compared with mature control.

only a 16% improvement from 2 days. Even after 8 weeks, the lymphocyte populations in the bone marrow of mature adult mice remained compromised, as evidenced by a $-24\% \pm 10\%$ and $-41\% \pm 14\%$ decline in the B and T-cells ($p < 0.05$). In the young adult mice, only the B-cells remained $-30\% \pm 24\%$ lower in the Irrad group when compared with the age-matched control at 8 weeks, whereas the T-cells had recovered to those levels measured in the age-matched control.

Age-dependent decline in hematopoietic stem cells and progenitor cells in the bone marrow

The phenotypic makeup of the hematopoietic stem cell and progenitor cell populations in the young and mature control mice were not statistically different, and at 2 days, the total number of LSK cells in the young Irrad mice were not significantly

different from the age-matched control. In direct contrast, LSK cells in the mature mice experienced an $-89\% \pm 7\%$ decline at 2 days, and therefore the LSK population in young Irrad mice was significantly greater than the older mice ($p < 0.05$; Fig. 6). At 10 days postirradiation, there was a $-61\% \pm 6\%$ and $-93 \pm 2\%$ decline in the total number of LSK cells in the young and mature Irrad mice compared with their age-matched controls, respectively ($p < 0.05$). As early as 2 days postirradiation, the SP-LSK cells had collapsed by $-87\% \pm 10\%$ and $-89\% \pm 5\%$ in the young and mature Irrad mice, respectively, and remained $-91\% \pm 4\%$ and $-87\% \pm 7\%$ depleted at 10 days compared with age-matched controls ($p < 0.05$). Although the SP-LSK population only reached partial recovery, remaining $-27\% \pm 16\%$ depleted in the young mice, the LSK subpopulations in the young Irrad had recovered to control levels by 8 weeks, whereas the LSK and SP-LSK cells were $-39\% \pm 14\%$ and $-28\% \pm 23\%$ suppressed in the bone marrow of the mature mice ($p < 0.05$).

Hematopoietic phenotypes in peripheral blood follows similar trend as the bone marrow

Irradiation-induced consequences to the lymphocyte phenotypes as measured in the peripheral blood were a clear sign of injury to both the B-cell and T-cell population. The absolute number of cells in the blood samples was difficult to determine because of cellular debris, so all data are represented with respect to phenotypes and not whole cell numbers. As early as 2 days postirradiation, there was a $-94\% \pm 4\%$ and $-96\% \pm 2\%$ decline in the T-cell phenotype of young and mature mice, respectively, when compared with their respective age-matched controls (Table 2). By 10 days, the T-cell phenotype in the Irrad young and mature mice was still $-82\% \pm 7\%$ and $-80\% \pm 11\%$ lower than age-matched controls. Eight weeks after irradiation, the T-cell phenotypic population was not significantly different in the young mice when compared with their age-matched controls. However, the recovery was hampered in the older animals, consistent with the depressed progenitor cell populations and leukocyte populations in the bone marrow, and the T-cell phenotype in the peripheral blood was $73\% \pm 17\%$ of age-matched control (NS).

B-cell populations in the peripheral blood also collapsed after irradiation, independent of animal age (Table 2). At 2 days, when compared with age-matched controls, the young Irrad mice showed a $-99\% \pm 1\%$ decline in the B-cell phenotype, recovering only slightly at 10 days to $-95\% \pm 1\%$ lower than control. Full recovery was not seen in the peripheral blood by 8 weeks: the percentage of B-cells in the peripheral blood was $-35\% \pm 25\%$ lower than controls in the young Irrad ($p < 0.05$) and $-46\% \pm 9\%$ lower in the older Irrad mice ($p < 0.05$).

Phenotypic shifts in myeloids in the peripheral blood were less severe than those measured in lymphocytes. Two days postirradiation, there was a $-38\% \pm 27\%$ decline of myeloids in the young Irrad mice ($p < 0.05$), and no decline whatsoever was measured in the mature Irrad mice. There were no other significant differences in the myeloid phenotype for the

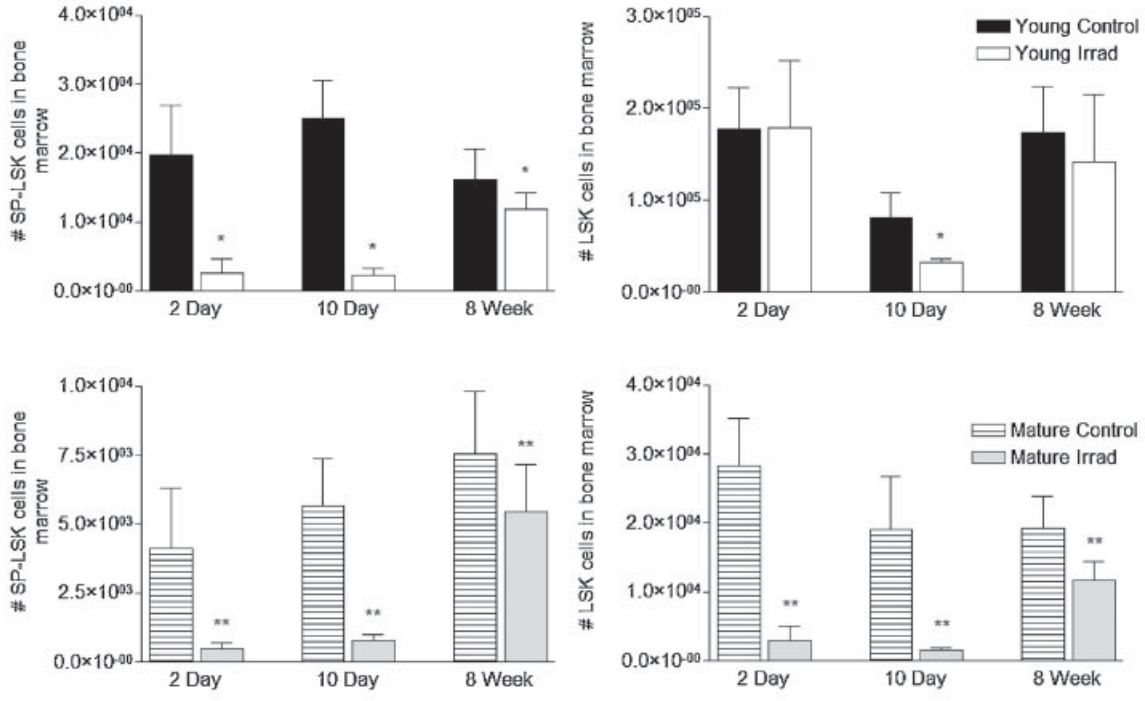

Fig. 6. Hematopoietic SP-LSK and LSK populations in the bone marrow of young and mature adult mice 2 days, 10 days, and 8 weeks after irradiation. Irradiation leads to drastic devastation of the SP-LSK cells after 2 days and 10 days, and even by 8 weeks does not reach complete recovery in the mice. The level of LSK cell depletion for irradiated mice at 2 days was dependent on age ($p < 0.05$). Young Irrad mice showed no level of LSK depletion until 10 days and recovered by 8 weeks, whereas the mature control mice experienced LSK devastation by 2 days and remained depleted at 8 weeks. $^{*}p < 0.05$ when compared with young age-matched control; $^{**}p < 0.05$ when compared with mature age-matched control.

Table 2. Phenotypic Makeup of the Peripheral Blood

		T-cells (CD4, CD8)	B-cells (B220)	Myeloid (Mac-1, Ly-6G/Ly-6C)
2 Days	Young control	6.43% ± 2.4%	22.81% ± 7.7%	4.50% ± 1.8%
	Young Irrad	0.38% ± 0.3%	0.19% ± 0.2%	2.77% ± 1.2%
	% Difference	−94.1 ± 4.1	−99.2 ± 0.9	−38.5 ± 26.8
	p value	0.000	0.000	0.044
	Mature control	4.50% ± 1.8%	14.22% ± 7.8%	2.62% ± 1.3%
	Mature Irrad	0.19% ± 0.1%	0.05% ± 0.0%	3.09% ± 1.3%
	% Difference	−95.8 ± 1.8	−99.6 ± 0.2	17.7 ± 48.9
	p value	0.000	0.000	0.446
10 Days	Young control	6.83% ± 2.7%	16.53% ± 6.5%	1.90% ± 0.6%
	Young Irrad	1.22% ± 0.5%	0.81% ± 0.2%	1.65% ± 0.6%
	% Difference	−82.2 ± 7.3	−95.1 ± 1.4	−13.4 ± 31.3
	p value	0.000	0.000	0.463
	Mature control	6.88% ± 2.0%	25.25% ± 6.4%	3.54% ± 1.0%
	Mature Irrad	1.37% ± 0.7%	0.56% ± 0.2%	5.91% ± 3.7%
	% Difference	−80.1 ± 10.8	−97.8 ± 0.9	67.0 ± 104.0
	p value	0.000	0.000	0.065
8 Weeks	Young control	8.29% ± 2.7%	24.24% ± 8.4%	8.25% ± 3.0%
	Young Irrad	7.20% ± 2.6%	15.87% ± 6.0%	7.52% ± 3.0%
	% Difference	−13.2 ± 30.8	−34.5 ± 24.8	−8.9 ± 35.9
	p value	0.443	0.046	0.639
	Mature control	2.97% ± 1.4%	16.10% ± 7.6%	2.94% ± 1.3%
	Mature Irrad	2.17% ± 0.5%	8.63% ± 1.4%	2.15% ± 0.5%
	% Difference	−26.9 ± 17.5	−46.4 ± 8.9	−27.1 ± 16.1
	p value	0.115	0.010	0.092

Note: The percentage of the T-cells, B-cells, and myeloids at 2 days, 10 days, and 8 weeks after irradiation are represented as mean ± SD. The B-cell and T-cell populations were rapidly depleted 2 days after irradiation, and even by 8 weeks, the B-cell population did not recover in the peripheral blood of the irradiated mice.

remainder of the study in the peripheral blood between the Irrad and the control.

Irradiation impact on bone morphology is independent of skeletal maturity

Two days postirradiation, osteoclast analysis revealed a 43% ± 35% increase in TRAP activity in the serum of mature Irrad mice compared with their age-matched controls ($p < 0.05$), with TRAP serum levels being 3.64 ± 0.89 U/L and 2.54 ± 0.54 U/L in the mature Irrad and mature control mice, respectively. High-resolution μCT analysis showed no significant decreases in bone mass and only slight changes in bone morphology in either young or mature mice at 2 days. However, by 10 days as compared with controls, marked changes were evident in both age groups, a collapse that persisted at least until the 8-week termination of the protocol. Ten days after irradiation, there was a −41% ± 12% and −39% ± 11% decline in the BV/TV in the young and mature mice ($p < 0.05$) compared with their age-matched controls, extending to a −45% ± 9% and −51% ± 6% decline at 8 weeks ($p < 0.05$; Fig. 7). By 8 weeks, microarchitectural quality of the bone remained severely damaged by irradiation, as reflected by the Tb.N being −34% ± 7% and −21% ± 3% ($p < 0.05$) lower in the young and mature Irrad mice, Conn.D being −81% ± 7% and −85% ± 7% lower than controls in the young and mature mice, and Tb.Sp being 56% ± 15% and 28% ± 6% greater than controls in the young and mature mice ($p < 0.05$).

At 10 days after irradiation, single-labeled surface was −48% ± 12% lower than that measured in age-matched controls ($p < 0.05$), whereas double-labeled surface, approximately 7% in the controls, was not detectable in the irradiated mice. Five weeks after irradiation, dynamic histomorphometry labels showed a 2.18 ± 0.18 μm/day MAR in the Irrad mice, indicating a 20% increase over the 1.82 ± 0.32 μm/day measured in age-matched controls ($p < 0.05$). BFR/BS was 0.36 ± 0.06 $\mu m^3/\mu m^2$/day in the Irrad, a 30% increase over the 0.28 ± 0.07 $\mu m^3/\mu m^2$/day measured in the age-matched controls ($p < 0.05$).

Discussion

Sublethal doses of radiation rapidly and severely damage the viability of the bone marrow, compromising the immune system of both young and old animals. A single sublethal dose of irradiation caused a severe collapse of the cellular populations within the bone marrow of young and skeletally mature mice, and although there was evidence of recovery by 8 weeks postinjury, the degree of that repair was dependent on the age of the animal and specific to the subpopulation of the precursor. It is clear, though, that certain subpopulations within the bone

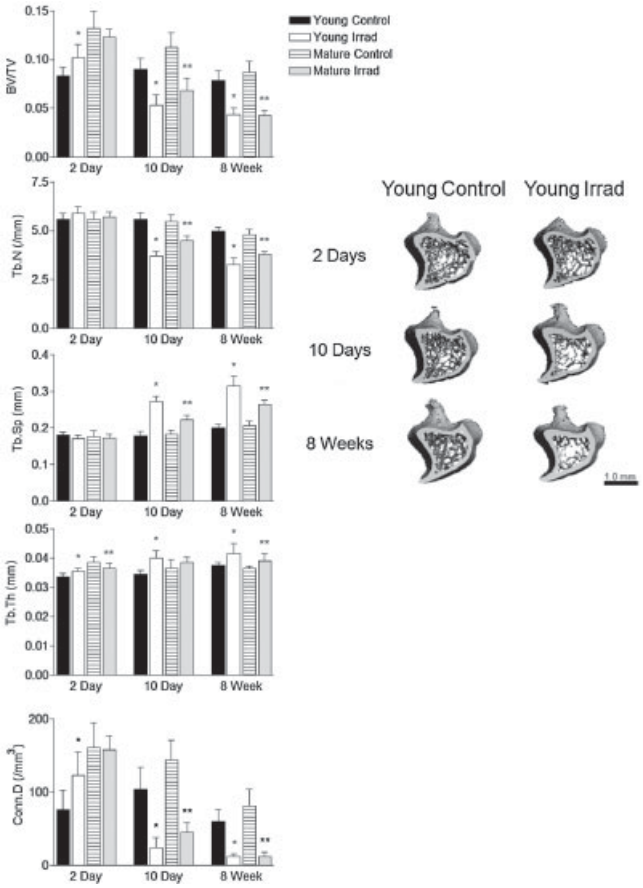

Fig. 7. Micro-CT analysis of the trabecular bone at 2 days, 10 days, and 8 weeks after irradiation in young and mature mice. The bone structural quality was devastated at 10 days after irradiation and did not repair itself at 8 weeks. $^{*}p < 0.05$ when compared with young control; $^{**}p < 0.05$ when compared with mature control.

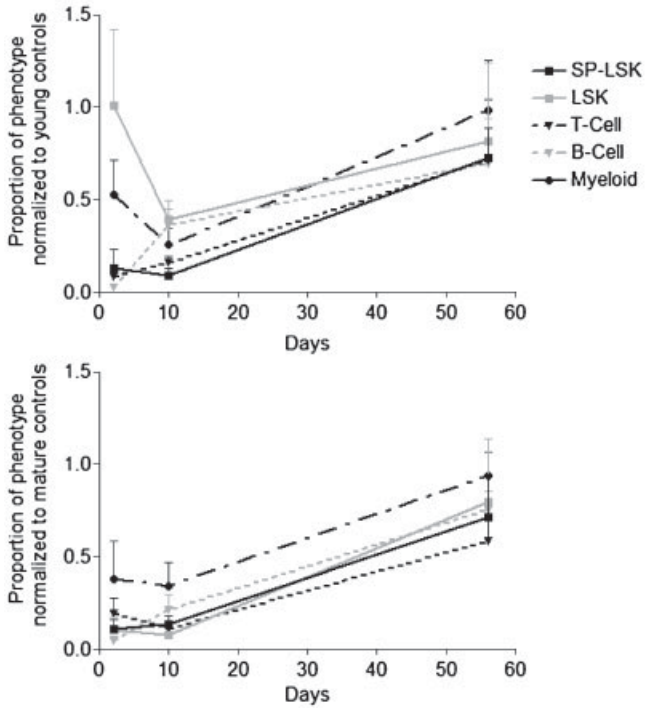

Fig. 8. Leukocyte phenotypes in the bone marrow of young (top) and mature (bottom) Irrad mice 2 days, 10 days, and 8 weeks after irradiation. The phenotypic composition of the cells in the bone marrow was altered after irradiation, and the decline of leukocytes was dependent on both the cell phenotype and the age of the mice. Although not necessarily reaching full recovery, all leukocyte populations showed improvement by 8 weeks.

marrow were more vulnerable to irradiation damage than others and slower to recover (Fig. 8).

The overall depletion of the cellular component of the bone marrow caused by irradiation appeared relatively similar between the young adult and skeletally mature adult mouse model. Initially, there was a sharp, approximately −90% decline of the SP-LSK cells, which persisted through to 10 days in both the young and mature mice, a drop similar to the lymphocyte population, regardless of age. Despite the rapid drop in the cell population in the bone marrow, it was evident that the younger mice were more effective and efficient in repairing the overall populations as well as the subpopulations, as evidenced by the rate of recovery in the LSK cells and T-cells. These age-dependent differences can readily be interpreted to suggest that the recovery of older animals is inherently slower partially because the DNA repair capacity is downregulated with increased age[21] and implies that the immune system will take significantly longer for complete restoration even though the phenotypic populations before irradiation for both the young and mature mice were not different from one another.

Despite the apparent collapse of overall viability of the bone marrow, it is interesting to note that as the SP-LSK cells declined in the bone marrow 2 days after irradiation, the LSK progenitor cells showed no immediate deficit in the younger mice. There are three possible explanations for their protection and/or retention: either the LSK cells are capable of restoring their cells within 2 days postirradiation, the SP-LSK cells are rapidly differentiating into LSK cells in an attempt to maintain homeostasis in the bone marrow, or the LSK cells in the young mice are more resilient and capable of lingering in the marrow longer before their eventual death as evidenced at 10 days (Fig. 6). In addition to being more quiescent, hematopoietic stem cells have been shown to be more resilient and therefore less susceptible to radiation-induced damage,[22,23] which further supports the initial sustainability of the LSK cells in young mice. By 10 days though, the LSK cells indicated a decline, one that was less severe than in the mature mice but was still −61% lower than the age-matched control. This could be attributed to the initiation of a possible leukocyte recovery by quickly differentiating into myeloid and lymphoid lineages to compensate for the overall loss in the bone marrow. It is also plausible that the LSK cells declined in number because the SP-LSK may have stopped differentiating toward their predetermined lineage to maintain their self-renewal capacity,[24] allowing for proper management of the compromised immune system. The mechanisms leading to a detriment to the LSK cells in mice at a young age may eventually hinder their HSC self-renewal capabilities and could obstruct molecular regulators necessary for the cell cycle,[25] ultimately compromising long-term health.

Not surprisingly, damage caused by irradiation was evident beyond the bone marrow. Similar hematological trends were apparent in both young and mature adult mice within the circulating blood. Extensive damage was evident in a range of physiologic systems, including the reduction in mass of the thymus and spleen evident at 2 days and 10 days, with the accompanying decrease in T and B-cells, and the severe deficit in the bone microarchitectural quality evident just 10 days postirradiation. The recovery of the spleen mass in the young mice by 8 weeks was not associated with a recovery of function, and a clear sign of systemic injury remained. This was evident by the inability of B-cells in all Irrad mice to return to normal levels in the peripheral blood. The decline in leukocytes in the peripheral blood was anticipated, as such drops are indicated by common clinical monitoring of bone marrow health after radiation therapy.[26] On the other hand, the age-independent collapse in bone quality and quantity, evident by 10 days after irradiation, was surprising because a vast depletion of cells in the bone marrow should hinder the bone turnover process, thus preventing bone resorption. The higher level of osteoclast activity (TRAP activity) and the marked suppression of bone formation (dynamic histomorphometry) certainly contributes to the loss of bone but may not fully account for the rapid collapse of bone architecture.

By 5 weeks after irradiation, histomorphometry showed an active turnover process, but it remained insufficient and did not achieve complete repair of the bone structure. The osteoblasts and osteoclasts appear to be returning to full function, allowing for bone formation and resorption, but have not yet become capable of overcoming the architectural deficit induced by radiation. Certainly, these data indicate that even with a fully recovered bone marrow population, the damaged bone morphology caused by radiation is much slower to repair and recover its strength, and in the case of those intentionally or inadvertently exposed to radiation, an increase in their risk of bone fractures may persist long after exposure.[27]

The age-independent decline in bone quality and quantity, evident as early as 10 days after irradiation, was initially presumed to be a cellular response caused by the decrease in bone formation (eg, the complete absence of double-labeled surface and a 48% decline in single-labeled surface) by 10 days, in concert with a doubling of TRAP activity as early as 2 days. Osteoclasts, the primary cells charged with the biologic excavation of bone, are derived from the hematopoietic stem cell lineages, a pool that had declined by $-87\% \pm 10\%$ and $-89\% \pm 5\%$ in the young and mature mice at 2 days, and remained as low as $-91\% \pm 4\%$ and $-87\% \pm 7\%$ at 10 days, suggesting that they were an unlikely source to elevate a robust population of resorptive cells. Surprisingly, the 43% increase in osteoclast activity at 2 days after irradiation showed an active resorption response even as the total number of cells in the bone marrow declined. Therefore, the effects of radiation are dependent on the phenotypic population in the bone marrow, and radiation is capable of upregulating specific populations within the hematopoietic lineage. Previous studies also report an initial increase in osteoclast activity per bone surface after a low dose (1 or 2 Gy) of X-ray and γ-irradiation.[28,29] Cumulative low doses (each less than 1 Gy) of γ-irradiation will still lead to a decline in the bone marrow population at a slower rate than a single dose, but they may prevent devastation to the bone architecture if in fact a nonbiologic effect is a contributing source of damage. Therefore, fractionated doses of radiation may be more beneficial to patients undergoing radiation therapy in regions exposed to skeletal tissue as shown by Overgaard's previous study that there is a decrease in rib fractures for women undergoing postmastectomy irradiation with lower doses of fractionation compared with higher doses with fewer fractions.[30] Thus, cumulative low fractionated doses for radiotherapeutic applications may hinder the bone devastation caused by an equivalent acute dose while still maintaining their efficacy.

To a degree, it was surprising that the level of bone devastation was similar between the young and mature mice, even at 8 weeks postexposure. One would also expect the mature mice to have poorer bone architectural parameters at 8 weeks after irradiation because of the inferior bone marrow population compared with the young adult mice. Unlike the young mice, the mature mice had a significantly lower LSK population and fewer total cells in the bone marrow at 8 weeks compared with their age-matched controls. We would therefore hypothesize an alteration in recovery for the bone architecture in young and mature mice, but this was not the case. Considering that the bone marrow cell pool was devastated, including those progenitors critical to bone resorption, the similarities in bone architectural decline suggests that this loss was independent of the repair of the hematopoietic lineages and perhaps even independent of a biologic process. Indeed, the amount of bone lost, so quickly, suggests that there may still be another cause of the decrease in mineral, such as that which might arise from cell-independent, physicochemical erosion enabled by radiation damage to the organic and inorganic constituents of the matrix.

Repair, as evident by elevated mineral apposition rate and bone formation rate per bone surface at 5 weeks postinjury, may initiate earlier with a bone marrow transplant, but the degree of organ damage as reflected by the loss of skeletal tissue is probably radiation dose dependent. Yet previous studies in C57BL/6 mice show whole bone marrow transplantation even 1 day after lethal irradiation does not prevent architectural damage and architectural recovery is never complete after irradiation.[31,32] Clinical studies also indicate that patients undergoing HSC transplantation experience their greatest bone loss within 6 months after irradiation,[33] while the bone marrow is repopulating. Therefore, the bone loss is initiated because there is increased osteoclast activity, the hematopoietic niche is not immediately reestablished after transplantation, or a nonbiologic process leads to decreased bone architectural quality. Indeed, the potential impact of long-term radiation exposure could lead to the slow destruction of the skeletal system leading to an increased risk of fracture, emphasizing the need for protective and reparative methods to preserve or reestablish structurally intact bone.

Numerous therapeutic methods are currently used to maintain and restore bone to prevent fractures. These include pharmacological approaches such as the administration of bisphosphonates, vitamin D, calcitonin, and hormone therapy.[5,34–36] Such therapies are targeting the bone though, rather than the

bone marrow, and the bone loss does not seem solely dependent on the activity of osteoclasts. Protecting the organ systems from failure is one approach that may be out of reach for the near future, but emphasizing new ways of restoring the progenitor population, rather than preserving the bone structure, might be a more effective means of restoring bone quality.

In conclusion, sublethal irradiation rapidly destroys a huge proportion of the bone marrow stem cell niche, but this destruction is not uniform across subpopulations in young and mature adult mice. Further, although the recovery of the bone marrow with time is fully enabled within two months in a young adult mouse, only partial recovery is achieved in older animals. The restoration of the marrow starkly preceded any signs of recovery of other physiologic systems, as indicated by the prolonged decline in skeletal morphology of both young and mature adult mice. Interventions that protect the bone marrow pool, or accelerate bone architectural restoration, could help to reduce health complications induced by medical or accidental radiation exposure and bone marrow disorders.

Disclosures

All the authors state that they have no conflicts of interest.

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