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ORIGINAL ARTICLE

TBI with lung dose reduction does not improve hematopoietic cell homing to BM during allogeneic transplantation

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To determine the effects of TBI dose, fractionation and lung shielding on hematopoietic stem cell homing to the BM, BM cells were extracted from tibiae and femurs of B6-green fluorescent protein (GFP) mice and transplanted into B6 mice. Recipient mice had either: (i) no radiation, (ii) single-dose TBI at 13.6 Gy, (iii) single-dose TBI at 13.6 Gy with reduced lung exposure to 0.4 Gy by shielding, (iv) split-dose TBI at 12 Gy to twice per day over 4 days or (v) split-dose TBI at 12 Gy to twice per day over 4 days with reduced lung exposure to 0.36 Gy by shielding. The last radiation exposure preceded tail vein injection by 4–6 h. Mice were killed after 18 h. The homing of GFP-positive, lineage-negative cells was not significantly improved in any irradiated group compared with control. The homing of GFP-positive, lineage-negative, Kit-positive cells was significantly worse in all irradiated groups. TBI does not improve the homing of lineage-negative donor BM cells to the recipient marrow. The homing of lineage-negative, Kit-positive donor BM cells was significantly worse following TBI, with or without lung dose reduction.

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Introduction

BM or PBST can be curative in the treatment of various human malignancies.[1–5] For SCT to be successful, the transplanted stem cells must home to the correct location within the BM.[6–8] In mouse models, irradiation has been found to alter the homing of stem cells.[9–11]

In humans, evidence suggests that TBI improves post-transplant survival compared with the use of chemotherapy alone.[12–14] Improved survival, however, may be mitigated by increased toxicity. In particular, lung toxicity has been shown to affect survival.[15–17] One strategy to reduce pulmonary toxicity is to simply reduce the lung dose directly by using lung shielding.[18] Several studies have described an association between lung dose reduction, reduced pulmonary-related mortality and improved OS.[19–21]

Specifically, our clinical experience showed that, compared with twice daily radiation alone, twice daily lung dose-reduced TBI can improve survival in a subset of patients.[19] Further analysis showed that this observed survival benefit of lung radiation dose reduction did not result from improved lung function.[22]

We wondered whether the observed survival benefit of twice daily TBI with lung radiation dose reduction may result from the altered homing of stem cells to the BM. To answer this question, experiments were carried out in mice to test the effects of twice daily lung dose-reduced TBI on the homing of hematopoietic stem and progenitor cells to the recipient BM.

Materials and methods

Animals

Normal C57BL/6 (B6) and the B6-enhanced green fluorescent protein (EGFP) transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained at NIH animal facilities under normal care and nutrition conditions. The EGFP mice had been backcrossed to B6 for 15 generations to ensure a relatively pure B6 genetic background. Similar combinations were used by others in studying hematopoietic cell and niche interactions.[23] All mice were used at 2–6 months of age and only male mice were used in the current study. All experiments were carried out under the aegis of a protocol approved by the National Cancer Institute Animal Care and Use Committee, and were in compliance with the Guide for the Care and Use of Laboratory Animal Resource, (1996) National Research Council.

TBI and lung shielding

To specifically study the effects of lung shielding, we used a Therapax DXT300 X-ray irradiator (Precision X-ray, Inc.,

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North Branford, CT, USA) using 2.0 mm Al Filtration (300 KVp) to irradiate B6 recipients at a dose rate of 2.35 Gy/min. We divided 25 B6 recipients into five groups of five mice each: (i) CON: no irradiation control, (ii) SD-NS: 13.6 Gy, single-dose TBI without lung shielding, (3) SD-BLS: 13.6 Gy single-dose TBI with bilateral lung shielding, (iv) BID-BLS: 12 Gy TBI in fractionated dose with bilateral lung shielding, (v) BID-NS: 12 Gy TBI in fractionated doses without lung shielding. Lung shielding consisted of a five half-value layer block, which reduced transmission to ~3% of the given dose. Fractionation, similar to the treatment of our clinical human cohort, consisted of twice daily radiation, 4-h apart at 1.5 Gy each time over 4 days for a total of 12 Gy. In this experiment, each recipient received 14.0×10^6 EGFP BM cells.

BM cells from EGFP donors were at 28×10^6 cells/ml in Iscove's Modified Dulbecco's Medium. Each recipient mouse received 0.5 ml donor cells, which were delivered through tail vein injection at 4–6 h after the last radiation treatment. Recipient mice were euthanized at 18 h after cell injection and the BM cells were collected and analyzed for donor hematopoietic cell homing.

FACS analysis and detection of hematopoietic cell homing in recipient mice

BM cells were extracted from the bilateral femurs and tibiae of recipient mice at 18 h after cell injection, and were filtered through a 90 μm nylon mesh. Cells were counted to calculate total BM cell recovery. All cells were stained with an Ab cocktail containing: Lin (CD3, CD4, CD8, CD11b, CD45R, Gr1, Ter119)-PE + CD34-PE-Cy5 + CD117-APC. The proportion and total number of GFP cells in each cell fraction were calculated to compute cell recovery (homing efficiency).

Procedures for flow cytometry were adapted as previously described.[24] In brief, BM cells were incubated in Gey's solution (130.68 mM NH_4Cl, 4.96 mM KCl, 0.82 mM Na_2HPO_4, 0.16 mM KH_2PO_4, 5.55 mM Dextrose, 1.03 mM $MgCl_2$, 0.28 mM $MgSO_4$, 1.53 mM $CaCl_2$ and 13.39 mM $NaHCO_3$) for 10 min on ice to lyse RBCs. After washing with a flow buffer (2.68 mM KCl, 1.62 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 7.69 mM NaN_3 and 1% BSA), cells were incubated with Ab mixtures on ice for 30 min. MoAbs for mouse CD3 (clone 145–2C11), CD4 (clone GK 1.5), CD8 (clone 53–6.72), CD11b (clone M1/70), CD45R (B220, clone RA3-6B2), CD117 (c-Kit, clone 2B8), erythroid cells (clone Ter119), granulocytes (Gr1/Ly6-G, clone RB6-8C5) and stem cell Ag 1 (Sca1, clone E13-161) were all from BD Biosciences (San Diego, CA, USA). Stained cells were analyzed on an LSRII flow cytometer using the FACSDiva software (Becton Dickinson, San Jose, CA, USA).

Statistics

Proportions of $EGFP^+$ and $EGFP^+Lin^-CD117^+$ cells in BM were multiplied by the total number of BM cells to calculate the total number of donor cells recovered in recipient BM as a percentage of $EGFP^+$ and $EGFP^+Lin^-CD117^+$ cell homing to recipient BM. The total number of BM cells per mouse was estimated assuming that one femur and one tibia contain 12.5% of total BM cells.[25]

Data were analyzed using JMP Statistical Discovery software (SAS Institute Inc., Cary, NC, USA), and were presented as mean with s.e. A P-value <0.05 was considered as statistically significant.

Results

Radiation reduces hematopoietic cell homing to host BM

Lung shielding did not improve the homing of any subset of GFP-positive cells to the BM of recipient mice following either the once daily or fractionated TBI.

To test the effect of lung shielding, we used X-rays to irradiate recipient mice as shown in Figure 1 with the lung

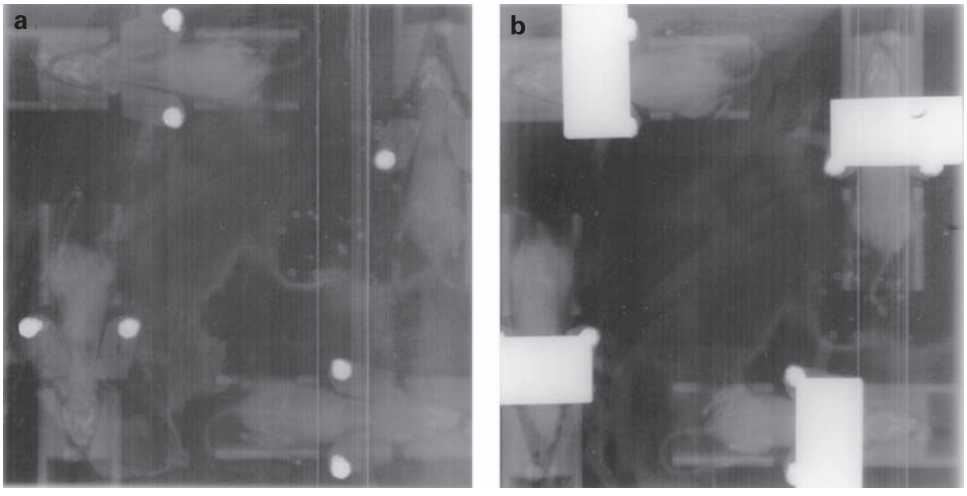

Figure 1 Mouse TBI and lung shielding. (**a**) Therapax DXT300 X-ray irradiator was used as the source of irradiation for all recipient mice used in the current study. Mice were anesthetized and placed on a specifically designed table. The effective X-ray area can accommodate four mice each time. Mice were irradiated for the entire body without shielding (**a**), or with lead shielding to reduce lung exposure to 3% of the effective dose (**b**).

shielding shown in Figure 1b. The EGFP donor mice that we used in the current study had higher levels of EGFP transgene expression in BM cells.

The top left panel of Figure 2 shows that 4% of recovered cells were lineage negative. The top right panel shows that 31% of the lineage-negative cells were also Kit^+. The lower two panels show that 98% of Lin^- cells and 99% of Lin^-Kit^+ BM cells from EGFP donor mice were positive for EGFP expression, the marker used to track cells in the recipient BM.

The homing of total GFP-positive BM cells (Figure 3, top), lineage-negative, GFP-positive BM cells (Figure 3, middle) and lineage-negative, Kit-positive, GFP-positive BM cells (Figure 3, bottom panel) was not significantly improved in any irradiated group compared with control. The homing of GFP-positive, lineage-negative cells was not improved by single-dose or fractionated TBI. In fact, fractionated radiation therapy showed a two- to fivefold decrease in donor hematopoietic cell homing compared with single-dose radiation (Figure 3).

As shown in the bottom panel of Figure 3, the homing of GFP-positive, lineage-negative, Kit-positive cells was significantly worse in all irradiated groups. Fractionated radiation therapy showed a fivefold decrease in homing compared with single-dose radiation. With either single dose or fractionated doses, lung shielding showed no beneficial effect for donor hematopoietic cells homing to recipient BM.

Discussion

This is the first study to show that lung shielding does not significantly alter the homing of transplanted stem cells following TBI. Moreover, this study shows that twice daily fractionated TBI does not improve the homing of donor BM cells to the recipient marrow. The homing of lineage-negative, as well as of the subset of lineage-negative, Kit-positive donor BM cells, was actually significantly worse following the twice daily over 4-day TBI regimen.

Though the true hematopoietic stem and progenitor cells have yet to be fully defined, multiple studies do show that transplanted cells capable of reconstituting hematopoiesis in lethally irradiated mice are lineage negative and Kit positive.[25–28] Cao *et al*.[25] showed that such transplanted stem cells can be recovered later from the femurs. Moreover, Cao *et al*. showed that it takes weeks for substantial proliferation to occur. In a magnetic resonance imaging study, Daldrup-Link *et al*.[29] showed that the majority of stem cells homing to the marrow occurs within 4–24 h after injection. As in our study, Kimura *et al*.[30] also used an 18-h time point to test the homing of stem cells without any substantial proliferation.

This study was designed to accentuate any radiation dose or fractionation-induced differences in the homing of lineage-negative, Kit-positive donor BM cells between groups by using a higher total dose in the single-fraction arms (13.6 Gy vs 12 Gy) and by limiting the lung dose to

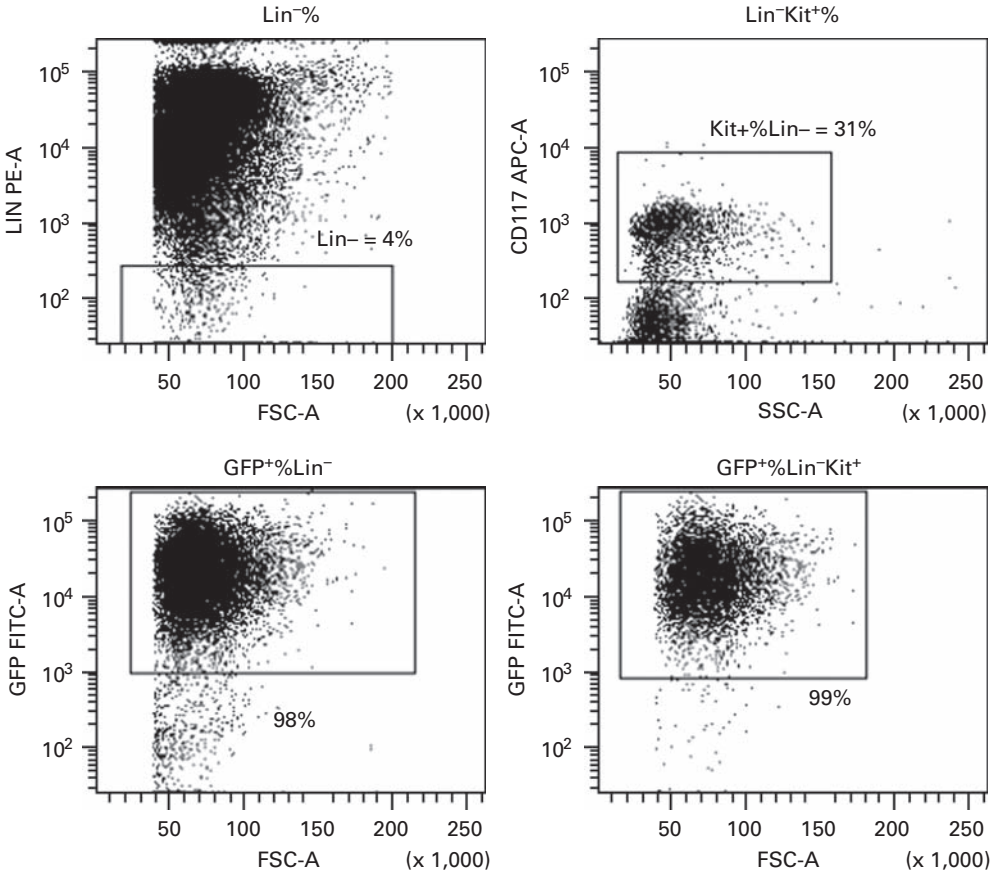

Figure 2 Flow cytometric analysis of donor BM cells. All recipient mice were then injected with BM cells from B6-green fluorescent protein (EGFP) donors for which the vast majority of Lin^- (98%) and Lin^-Kit^+ (99%) of donor BM cells were $EGFP^+$.

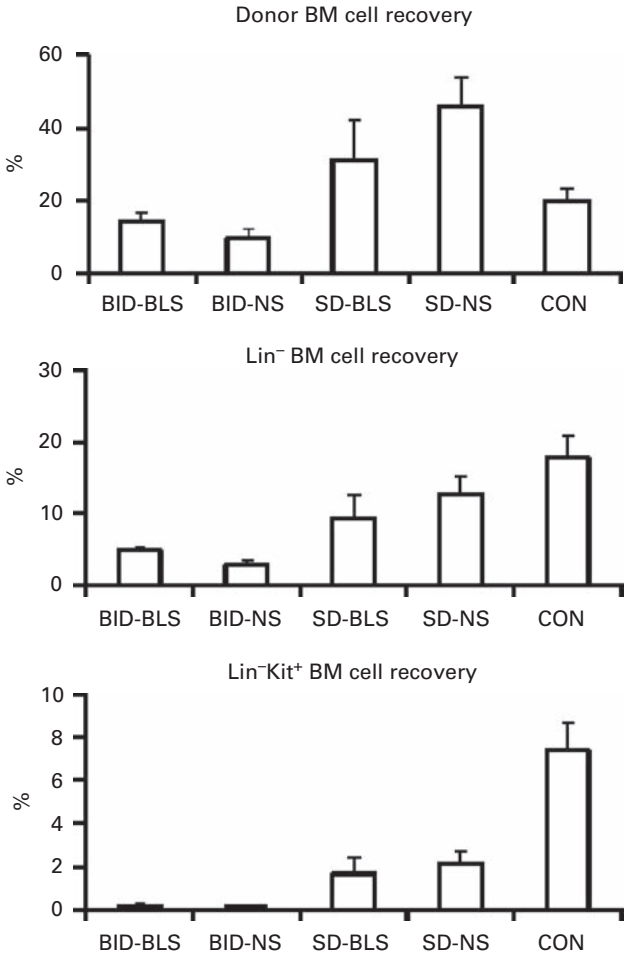

Figure 3 Effect of fraction dose and lung shielding on hematopoietic cell homing. Homing of enhanced green fluorescent protein donor Lin^-Kit^+ cell to recipient BM was shown as percentages of total BM (top), Lin^- (middle) and Lin^-Kit^+ (bottom) cell recovery in recipient BM that were computed and presented as means with s.e. bars, respectively, showing that irradiation reduces hematopoietic cell homing to recipient BM and fractionated dose further reduces cell homing, with or without lung shielding. CON, no irradiation control; SD-NS, 13.6 Gy) single-dose TBI without lung shielding; SD-BLS, 13.6 Gy single-dose TBI with bilateral lung shielding; BID-BLS, 12 Gy TBI in fractionated dose with bilateral lung shielding; BID-NS, 12 Gy TBI.

3% (compared with 50% in the clinical setting) in the lung shielding groups.

Despite these attempts to accentuate differences that favored the fractionated arms, the fact that fractionated radiation therapy caused a decrease in donor hematopoietic cell homing compared with single-dose radiation is a fresh observation for which we lack a definitive explanation. Kovacs *et al*.[31] reported that fractionated radiation therapy generates two forms of dose-dependent damage in the marrow. In the first form of dose-dependent damage, an early lesion arises in the blood-forming subpopulations. In the second form of dose-dependent damage, a delayed lesion arises that involves the persistent expression of a dysfunctional microenvironmental phenotype. On the basis of these data, we speculate that fractionated doses of irradiation may have caused more damage to the host stromal environment than single-dose irradiation, thus hampering the homing of infused donor hematopoietic stem and progenitor cells.

It is noted that the dose rate of 2.35 Gy/min used in this study was different from the clinical dose rate of 0.12 Gy/min. However, Colis *et al*. showed that the TBI dose rate, varying from 0.01 to 5.85 Gy/min, did not affect the homing of stem cells to the BM.[10]

Our data showing decreased hematopoietic stem and progenitor cell homing after TBI echo those of Collis *et al*.,[10] which showed an approximately fivefold reduction in lineage-negative hematopoietic cell homing to the BM compared with unirradiated control animals. Similarly, by comparing radiated with non-radiated mice, Plett *et al*.[11] found a 5–30 fold reduction in the homing of stem cells capable of long-term hematopoiesis in secondary recipients.

Conversely, our and the aforementioned data contravene the experience of Bastianutto *et al*.,[32] who found that local irradiation does induce a fourfold greater homing of hematopoietic stem cells to the locally irradiated BM. In this experiment, however, TBI was not performed in any group. Therefore, it remains possible that the effects of isolated local irradiation are quite different from TBI in the induction of hematopoietic stem cell homing.

Overall, multiple publications and our data support decreased hematopoietic stem and progenitor cell homing after TBI.[10,11] However, these data must be interpreted with caution. Human data from our group and others suggest that the stem cell dose is not linearly related to survival. In fact, many series have shown that above an optimum stem cell dose ($\sim 8–10 \times 10^6$ cells per kg) the probability of mortality actually increases with increasing stem cell dose.[33–35] Given this fact that (above an optimum level) increased stem cell dose actually decreases survival, one cannot simply assume that the decreased stem cell homing seen following TBI will necessarily diminish survival.

Multiple questions remain about the homing of lineage-negative, Kit-positive cells following TBI. Earlier studies have shown that some fraction of transplanted stem cells do home to the spleen[36–39] and/or lungs.[36,39] Therefore, one may posit that TBI with or without lung shielding may increase homing to these other sites. However, a prior iteration of these experiments, similar to those detailed here but with female mice (data not shown), showed consistently less homing—on a similar scale as shown for BM—of GFP-positive, lineage-negative, Kit-positive cells to the spleen, lung and BM following TBI with or without lung shielding. Thus, it appears that TBI, with or without lung shielding, decreases the homing of lineage-negative, Kit-positive cells to the BM, spleen and lung.

Future directions

Stem cell transplant is a complicated process with many effective approaches (for example, types of chemotherapy used, performed with or without TBI) and a multitude of variations (for example, dose of chemotherapy used, single-fraction or multiple-fraction TBI with or without lung dose reduction, low dose.) The absence of a definitively superior approach suggests that no optimum regimen will soon emerge. Therefore, each approach will need to be optimized. Given the complexity of the successful transplant,

we advocate making only those limited changes to the transplant regimen necessary to test the hypothesis while holding all other variables constant.

We have found no preclinical evidence that TBI with bilateral lung shielding improves stem cell homing. The mechanisms that explain the survival benefit seen in a subset of patients treated with lung dose-reduced, twice daily TBI remain unknown.[19] However, given the success of lung dose reduction and absence of any homing advantage in preclinical models, we are pursuing lower doses of TBI in selected older patients.

Conclusion

The current data add to the existing literature by showing that the survival benefit seen with lung dose-reduced, twice daily TBI[19] likely does not arise from the improved homing of hematopoietic stem and progenitor cell homing to the BM. However, no definite conclusion can be reached about the homing of the true stem cell subset until that subset is adequately defined.

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