Host marrow stem cell potential and engraftability at varying times after low-dose whole-body irradiation

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Introduction

Marrow stem cells home to and engraft in normal nonmyeloablated hosts.1-6 The final percent donor chimerism is determined by the ratio of donor to host stem cells rather than the availability of open marrow space; thus, in essence, stem cell competition is the determinant of the percent donor chimerism in syngeneic marrow transplantation.

Engraftment into normal hosts was reported as early as the 1960s although its significance was not stressed.1 Later studies using various cell tracking techniques showed that variable, and at times significant, short-term chimerism could be obtained by simply infusing distinguishable donor cells into normal nonmyeloablated mice.2-5 Stewart et al6 extended these observations showing long-term (> 2 years) multilineage engraftment of male cells in female marrow, spleen, and thymus. Further studies showed that nonproliferating quiescent stem cells engrafted into normal hosts and that cell dose-response curves could be established.7-9 Mathematical modeling of engraftment into normal hosts, assuming either stem cell replacement or stem cell augmentation, indicated that virtually all infused stem cells had engrafted; final host:donor marrow cell percentages 8 weeks after cell injection at week 0, 6, 12, or 24 were 68% ± 12%, 45% ± 15%, 51% ± 12%, or 20% ± 8%, respectively. Eight-week engraftment levels in nonirradiated hosts average 7.7%. Conversely, engraftable stem cells measured at 8 weeks postengraftment in 1 Gy−exposed hosts were reduced to 8.6% ± 3% of nonirradiated mice at time 0, 35% ± 12% 6 weeks later, 49% ± 10% at 3 months, and 21% ± 7% at 6 months. Engraftment was still increased and stem cell decreased 1 year after 1 Gy. Furthermore, the primary cells transplanted into 1 Gy hosts can be serially transplanted, and the predominant effect of 1 Gy is directly on engrafting stem cells and not through accessory cells. These data show that transplantation in 1 Gy mice may be delayed until recovery of hematopoiesis, suggesting strategies in allogeneic transplantation to avoid the adverse effects of cytokine storm. The incomplete recovery of engraftable stem cells out to 12 months indicates that stem cell expansion, especially in patients previously treated with radiomimetic drugs, may not be feasible. (Blood. 2001;98:1246-1251)
cells and diminishment of the host’s ability to accept and express a marrow graft at various times after 1 Gy WBI. In addition, the ability of marrow cells engrafted in 1 Gy hosts to serial transplantation was unknown, as was the possibility that accessory cell ablation played a role in the apparent stem cell effect of 1 Gy WBI. These issues are also addressed.

Materials and methods

Mice

Six- to 8-week-old BALB/c (H-2b) mice (Taconic Farms, Germantown, NY) were housed in a conventional clean facility for at least one week prior to experimental use. Mice received mouse chow and acidified water ad libitum.

Marrow cell suspension

BALB/c mice were killed by cervical dislocation, and bone marrow cells were collected from tibiae and femurs. Cells were flushed from femurs and tibiae with phosphate-buffered saline and filtered through a 40-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ). The cells were washed once in phosphate-buffered saline, counted in crystal violet, and resuspended for injection in phosphate-buffered saline.

Transplantation

Female BALB/c mice were exposed to 1 Gy WBI (Gammacell, 0.89-1.02 Gy/min). These irradiated mice were then infused with 40 x 10^6 normal male BALB/c marrow cells 3 to 4 hours after host irradiation or, alternatively, as illustrated in Figure 1, 6, 12, and 24 weeks after irradiation. These mice were then evaluated 8 weeks after cell infusion for engraftment by determining the percentage of male cells in female marrow using a fluorescent in situ hybridization (FISH) technique with a Y painting probe (see below) (Figure 1).

These studies were planned to establish host engraftability as a final percent of donor cells in host marrow at different times postirradiation. We also evaluated host engraftable stem cell content at different time points after irradiation. In these experiments, male BALB/c mice were exposed to 1 Gy WBI, and then 3 to 4 hours or 6, 12, and 24 weeks after irradiation marrow cells were harvested; the ability to engraft and proliferate in 1 Gy–treated female BALB/c mice was determined 8 weeks after cell infusion (Figure 2).

An experiment was designed to determine whether cells engrafted into a 1 Gy–treated host could be serially transplanted, indicating the stem cell nature of the original transplant (Figure 3). Here we initially engrafted 40 x 10^6 normal male BALB/c marrow cells into 0- or 1 Gy–treated female BALB/c hosts. We also evaluated similar transplants with 1 Gy–exposed donor marrow. Eight weeks after the initial cell infusion, 20 x 10^6 chimeric (male plus female) marrow cells from the primarily engrafted group were reinfused into 10 Gy (1000 cGy)–exposed female mice. These groups were also followed for 8-week engraftment, evaluated, and 20 x 10^6 chimeric cells infused into 10 Gy–treated tertiary female hosts. Engraftability was determined 8 weeks after each infusion by FISH for percent male cells (see below). These experiments are outlined in Figure 3.

Accessory cell effects

We designed experiments to determine whether irradiation could be directly affecting the stem cells or indirectly affecting them through accessory or helper cells. We have approached this in 2 ways: (1) A total of 40 x 10^6 nonirradiated female marrow cells were admixed with 40 x 10^6 1 Gy–exposed male cells, and the mixture was compared with nonirradiated male cells for engraftment into both nonirradiated and 1 Gy–exposed hosts. (2) We purified Lin<sup>−</sup> Hoechst 33342<sub>low</sub> Rhodamine<sub>low</sub> (see below) stem cells and then evaluated these cells after 0 or 1 Gy irradiation for 8-week engraftment into 1 Gy–exposed hosts.

FISH

The FISH technique identifies individual cells that contain the Y chromosome. Cells that are positive are male, and cells that are negative are female. Essentially 100% of male cells are labeled and 100% female cells are negative. Cells are fixed in Carnoy fixative (75% methanol/25% acetic acid) once for 20 minutes and then transferred to Eppendorf tubes for long-term storage at −20°C. The cell preparation is washed in fresh Carnoy fixative.
once and then dropped on ethanol for 1 hour. The air-dried slides are then permeabilized with 0.1 N HCl/0.05% Triton X-100 at room temperature for 10 minutes; washed with 2 × SSC; denatured in 70% formamide in 2 × SSC at 70°C for 3 minutes; dehydrated in ice-cold 70%, 85%, and 100% ethanol, each for 2 minutes; and hybridized with a digoxigenin-labeled murine Y chromosome painting probe at 37°C overnight. Unbound probe is removed by stringent washing in 50% formamide in 2 × SSC at 45°C for 5 minutes and then 2 × SSC at room temperature. The slides are blocked using a blocking buffer consisting of 5% fetal calf serum, 5% nonfat milk (Shaw’s, Bridgewater, MA), and 0.05% Triton X-100 (Sigma, St Louis, MO) in 4 × SSC for 30 minutes. Detection of digoxigenin is done using antidigoxigenin fluorescein isothiocyanate–labeled Fab fragments (Boehringer Mannheim, Indianapolis, IN) incubated in the dark for 30 minutes. Nonbound antibody is removed through 3 light-protected washings in 4 × SSC containing 0.05% Triton X-100, each for 5 minutes. Finally, slides are mounted in the antifade media vectashield (Vector, Newcastle, United Kingdom) with 0.4 μM DAPI (4′,6-diamidino-2-phenylindole·2HCl) (Sigma), which counterstained the DNA. Specific positive signal is confirmed under the UV microscope by a visual check at excitation and emission wavelengths other than that of fluorescein isothiocyanate. Positive and negative controls consisted of male and female slide preparations, respectively. The percentage of positive male cells in female hosts is determined by male cell numbers divided by the total cells counted. For each sample, at least 100 cells were counted under the UV microscope.

Isolation of Lin− Hoescht 33342low Rhodaminehigh stem cells
Bone marrow was isolated from iliac bones, femur, and tibia of BALB/c mice 6 to 8 weeks of age. A low-density fraction (< 1.077 g/cm3) was isolated on Nycoprep 1.077A (Accurate Chemical and Scientific, Westbury, NY). These cells were lineage-depleted with the following antibodies: Ter119, B220, Mac-1, Nycoprep 1.077A (Accurate Chemical and Scientific, Westbury, NY). These cells 1.077 g/cm 2 ) was isolated on

| Table 1. Male BALB/c marrow engraftment into female BALB/c mice at different times after 1 Gy whole body irradiation |
|-----------------|-------|---------------|-------|---------------|-------|---------------|-------|---------------|-------|
| Experiment no. | Gy    | No. | Mean ± SD    | No. | Mean ± SD    | No. | Mean ± SD    | No. | Mean ± SD    |
| 1              | 0     | 5   | 8.7 ± 3.4    | 4   | 6.3 ± 4.6     | —   | —             | —   | —             |
| 2              | 1     | 5   | 65.3 ± 8.4   | 5   | 42.7 ± 9.9    | 2   | 61.8 ± 9.0    | —   | —             |
| 3              | 0     | 5   | 10.3 ± 4.7   | 5   | 10.7 ± 3.8    | 5   | 7.9 ± 1.6     | —   | —             |
| 4              | 0     | 5   | 7.7 ± 15.8   | 5   | 64.6 ± 10.0   | 5   | 46.3 ± 9.7    | —   | —             |
| 5              | 1     | 5   | 68.0 ± 6.6   | 5   | 39.3 ± 11.9   | 4   | 50.2 ± 13.7   | —   | —             |

Engraftment determined by FISH (see “Materials and methods”).
shown in Table 1. Expressed as a fold enhancement over engraftment seen in nonirradiated age-matched control hosts, engraftment at 0, 6, 12, and 24 weeks was enhanced 10.1 ± 3.6, 6.7 ± 0.8, 7.7 ± 3.0, and 5.75 ± 0.0, respectively (Figure 5). These enhancements were all statistically significant at *P* < .02. When female hosts were irradiated with 1 Gy immediately or 12 months before transplantation of 40 × 10^6 male marrow cells, the engraftment at 8 weeks was 71.1% ± 3.4% for time 0 and 23.2% ± 0.2% at 12 months, whereas the nonirradiated age-matched controls had 7.2% ± 0.7% and 5.4% ± 2.1%, respectively (Figure 6). This represents a 9.9 ± 1.4-fold and 4.3 ± 1.7-fold enhancement of engraftment over controls, respectively.

The residual host engraftable stem cells, as measured at 8 weeks after cell infusion, were analyzed immediately and at 6, 12, and 24 weeks after 1 Gy exposure. As previously reported, engraftable stem cells were markedly suppressed when determined immediately after 1 Gy WBI. Engraftment capacity of marrow from 1 Gy–exposed mice was only 8.62% ± 2.7% of the engraftment capacity of marrow from nonirradiated BALB/c mice (Figure 7). This represents a 7.9-fold decrease in stem cell engraftment capacity (Figure 8). When marrow stem cells were assayed 6 weeks after 1 Gy engraftment, capacity was 34.5% ± 12% of age-matched nonirradiated marrow. This represented a 2.1-fold decrease in engraftment capacity. At 12 weeks postirradiation, engraftment capacity had recovered to 48.5% ± 10% of nonirradiated controls. In a small number of mice evaluated at 6 months after 1 Gy engraftment, capacity was still suppressed (20.9% ± 6.8% of nonirradiated control), with a 1.5-fold decrease compared with age-matched control. Engraftment of irradiated marrow at 6 and 12 weeks was significantly higher than that at 0 weeks (Wilcoxon rank sum test, *P* < .001) and showed an increasing time trend (Cuzick nonparametric trend test, *P* < .01). When male donors were irradiated with 1 Gy immediately or 12 months before bone marrow harvest and transplantation of 40 × 10^6 cells into 1 Gy–treated female recipients, the engraftment at 8 weeks was 10.5% ± 1.2% for time 0 and 33.6% ± 4.6% at 12 months, whereas the nonirradiated bone marrow control group had 71.1% ± 3.4% at time 0. There was no age-matched control for this experiment (Figure 9).

To further evaluate the stem cell nature of cells engrafting into 1 Gy–treated hosts, we evaluated the ability of marrow harvested 8 weeks postengraftment of 40 × 10^6 male BALB/c cells into 1 Gy–treated female BALB/c hosts to serially repopulate in 1 Gy–treated secondary and tertiary hosts at 8-week intervals (Figure 3). Engraftment in each case was determined by FISH, and for the secondary and tertiary transplants 20 × 10^6 cells were infused. The groups for the initial experiment consisted of (1) 40 × 10^6 nonirradiated male BALB/c marrow cells infused into nonirradiated hosts (designated 0 Gy/0 Gy); (2) 40 × 10^6 1 Gy–exposed marrow cells infused into nonirradiated hosts (1 Gy/0 Gy); (3) 40 × 10^6 1 Gy–exposed marrow cells infused into 1 Gy–exposed hosts (1 Gy/1 Gy); and (4) 40 × 10^6 nonirradiated marrow cells infused into 1 Gy–exposed hosts (0 Gy/1 Gy). At 8 weeks after this primary transplant, 20 × 10^6 chimeric cells were harvested and then infused into 5 secondary 10 Gy–exposed female BALB/c recipients; this was again repeated in 8 weeks, and engraftment assessed 8 weeks after each transplant. In the primary transplant, there were 55.6% ± 6% male cells in marrow 8 weeks after transplantation.
This was the marrow inoculum for the 20 × 10^6 cells used for the secondary transplant. (Figure 10).

These data show the stem cell nature of the male cells engrafting into 1 Gy hosts but also show that with serial transplantation the male cells seem to compete less well with the female cells in the pooled marrow mixtures, possibly indicating a deleterious effect of the initial engraftment. It is unlikely that secondary or tertiary recipient female cells contributed significantly to these populations 8 weeks after 10 Gy conditioning.

The possibility existed that treatment with 1 Gy reduced marrow stem cell engraftability by altering non–stem cell facilitator or stimulatory cells rather than directly affecting the stem cells. Thus, we evaluated whether adding 40 × 10^6 nonirradiated female cells to the 40 × 10^6 1 Gy–exposed male cells and infusing these into female BALB/c hosts would enhance engraftment of the male cells. We evaluated groups of mice in which hosts and donors were nonirradiated (0 Gy/0 Gy), in which donors were irradiated but not hosts (1 Gy/0 Gy), in which donors and hosts were irradiated (1 Gy/1 Gy) and, finally, in which donors were nonirradiated and hosts irradiated (0 Gy/1 Gy). In each instance, results for 8-week engraftment were determined in cohorts of mice either receiving only 40 × 10^6 male cells (without female) or 40 × 10^6 male and 40 × 10^6 female marrow cells (with female) (Figure 11). One Gy exposure of host marrow resulted in the expected decrease in male engraftment. Additional female marrow, rather than increasing engraftment, resulted in an apparent dilutional decrease of male engraftment. This indicated that the 1 Gy WBI was probably acting directly on male-engraftable stem cells.

Furthermore, we evaluated the direct effect of 1 Gy irradiation at the stem cell level. Purified Lin^-Hoechstlow Rhodamine123low–sorted stem cells from male BALB/c mice (1.8 × 10^6 per mouse) were injected intravenously in 1 Gy–treated female BALB/c recipients. Prior to transplantation, half of the sorted stem cells were exposed to 1 Gy irradiation, and the other half received no treatment. Engraftment was measured in bone marrow 8 weeks after transplantation with FISH using a Y-chromosome painting probe. Cells were counted using a Rhodamine filter to give the percentage of engraftment. Mean ± SE is shown (n = 5, P = .001).

**Discussion**

Engraftment of BALB/c male marrow cells into 1 Gy–treated female hosts has validated the model of stem cell engraftment based on host donor stem cell competition forwarded by studies on transplantation in nonmyeloablated hosts.

The present result extend information on this model, showing that the engrafting cells have secondary and tertiary engrafting capacity, ie, they are true stem cells, although the relative decrease in male chimerism over time suggests that male stem cells may have been damaged by the initial transplant. In addition, the question of whether the defective engraftment of marrow from mice exposed to 1 Gy WBI could be due to a depletion of accessory cells or enrichment of inhibitory cells rather than a direct effect on engrafting stem cells was answered. Addition of normal female BALB/c marrow to irradiated male BALB/c marrow did not increase engraftment but, rather, resulted in a dilutional decrease in male cell engraftment. The 1 Gy treatment of purified murine Lin^-Hoechstlow Rhodamine123low stem cells free of accessory cells resulted in a significant decrease of engraftability, confirming a direct toxic effect of irradiation on the engraftability of purified hematopoietic stem cells independently from the accompanying cells.
In previous studies, we have shown that peripheral blood counts have returned to normal by 2 weeks after 1 Gy. Thus, the present data suggest interesting strategies for allogeneic transplantation. The “cytokine storm” seen immediately after high-dose chemoradiotherapy exerts an adverse effect on graft-versus-host disease. Perhaps delaying allogeneic transplantation in 1 Gy–treated hosts to a time when steady-state lymphohematopoiesis has been achieved may allow for less graft-versus-host disease and superior results with allogeneic marrow transplantation. Work by Xun and colleagues has, in fact, shown that recovery in BALB/c mice markedly reduced acute graft-versus-host mortality in these hosts after major histoincompatible transplantation. Perhaps a further delay of 2 to 3 weeks postirradiation with a lower irradiation dose might further improve on these results. Whether alteration of the timing of transplantation in a syngeneic transplant setting employing low-dose irradiation might improve engraftment results remains an open question.

The present results with host stem cell depletion and engraftment immediately after 1 Gy remain consistent with the concept of stem cell competition determining donor chimerism. However, the observation that stem cell recovery after 1 Gy was more rapid and complete than alterations in host engraftability suggests that factors in addition to straight stem cell competition may be operative in the 1 Gy–exposed host. Although studies by Hendrikx et al suggest that high-dose irradiation may actually impair stem cell homing, it is possible that lower-dose host irradiation may facilitate homing by altering cytokines, cytokine receptors, or adhesion proteins—to cite a noninclusive list.

Exposure of BALB/c mice to 1 Gy resulted in a profound deficit of engraftable stem cells as measured in 1 Gy hosts. Although there was recovery, this deficit persisted out to 6 months (and probably out to 1 year) postirradiation. This suggests that significant stem cell expansion, an elusive goal at best in vitro, may be very difficult in animals or patients previously treated with radiation or radiomimetic agents and is consistent with data indicating the existence of a proliferative defect in murine primitive hematopoietic stem cells after exposure of mice to higher levels of ionizing radiation or to various chemotherapeutic agents. This also resonates with data indicating a decline of stem cell capacity on transplantation and telomeric DNA loss in in vitro “expansion” cultures. However, the in vitro data indicating difficulty in expanding long-term engraftable stem cells need to be balanced against recent reports of in vivo expansion of long-term engraftable stem cells in the presence of thrombopoietin, horse serum, and murine stroma.

References

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