Engraftment of Bone Marrow Cells Into Normal Unprepared Hosts: Effects of 5-Fluorouracil and Cell Cycle Status

By Hayley S. Ramshaw, Sudhir S. Rao, Rowena B. Crittenden, Stefan O. Peters, Heinz-Ulrich Weier, and Peter J. Quesenberry

Bone marrow from animals treated with 5-fluorouracil (5FU) competes equally with normal marrow when assessed in vivo in an irradiated mouse, but shows markedly defective engraftment when transplanted into noncytoablated hosts. Using Southern Blot analysis and a Y-chromosome specific probe, we determined the level of engraftment of male donor cells in the bone marrow, spleen, and thymus of unprepared female hosts. We have confirmed the defective engraftment of marrow harvested 6 days after 5FU (FU-6) and transplanted into unprepared hosts and shown that this defect is transient; by 35 days after 5FU (FU-35), engraftment has returned to levels seen with normal marrow. FU-6 marrow represents an actively cycling population of stem cells, and we hypothesize that the cycle status of the stem cell may relate to its capacity to engraft in the nonirradiated host. Accordingly, we have evaluated the cycle status of engrafting normal and FU-6 marrow into normal hosts using an in vivo hydroxyurea technique. We have shown that those cells engrafting from normal marrow and over 70% of the cells engrafting from FU-6 marrow were quiescent, demonstrating no killing with hydroxyurea. We have also used fluorescent in situ hybridization (FISH) analysis with a Y-chromosome probe and demonstrated that normal and post-5FU engraftment patterns in peripheral blood were similar to those seen in bone marrow, spleen, and thymus. Altogether these data indicate that cells engrafting in normal, unprepared hosts are dormant, and the defect that occurs after 5FU is concomitant with the induction of these cells to transit the cell cycle.

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have investigated the duration of the post-5FU engraftment defect and the cell cycle status of normal and post-5FU marrow cells engrafting into normal unirradiated hosts, with the underlying hypothesis that engraftment potential of hematopoietic renewel cells in the unprepared mouse is linked to the cell cycle status of these cells.

MATERIALS AND METHODS

Mice. BALB/c mice were purchased from Charles River Laboratories, Wilmington, MA and were maintained in virus-free conditions at the Animal Facility at the University of Massachusetts Medical Center, Biotech II building. All animals were given food and acidified water ad libitum. Mice were purchased at either 16 to 18 g or 18 to 22 g and were housed for a minimum of 1 week before experimental use.

Transplantation of murine marrow from 5FU-treated donors into normal untreated hosts. BALB/c female mice were transplanted for 5 consecutive days with intravenous injections of cells from two tibiae and two femurs from male BALB/c mice or with phosphate-buffered saline (PBS) (GIBCO-BRL, Bethesda, MD). Donor male mice were untreated or injected via the tail vein with 5FU (150 mg/kg) (SoloPak Laboratories, Franklin Park, IL) on day 0, then bone marrow was procured 6 or 35 days after treatment (FU-6 and FU-35, respectively). Volumes for injections were 0.5 mL per transfusion. The percentage of male marrow in total host marrow cells was determined using Southern blot analysis with the Y-chromosome-specific pY2 probe (a gift from Dr Ihor Lemischka, Princeton University, Princeton, NJ). Mice were killed at 7 or 10 weeks posttransplant. Southern blot analysis was also used to assess the percentage of male marrow in spleen and thymus tissues. Fluorescent in situ hybridization (FISH) was used to quantitate engraftment on a single cell basis in peripheral blood.

Transplantation of murine marrow from hydroxyurea-treated donors into normal hosts. BALB/c female mice were transplanted intravenously for 5 consecutive days with two tibiae and two femurs from male BALB/c mice that had been treated with hydroxyurea (900 mg/kg) (Sigma Chemical Co, St Louis, MO) or saline 2 hours before killing. Control recipient female mice received 0.5 mL PBS (GIBCO-BRL) for 5 days. Percentage male cells in bone marrow, spleen, and thymus were analyzed at 12 weeks posttransplant by Southern blotting.

BALB/c female mice were also transplanted via tail vein injection for 5 consecutive days with cells from two tibiae and two femurs from male BALB/c mice that had been treated with 5FU (150 mg/kg) (SoloPak Laboratories) on day 0 and either hydroxyurea (Sigma) or saline on day 6. Donor mice, FU-6, were given hydroxyurea (900 mg/kg) or saline 2 hours before killing. Control recipient female mice received 0.5 mL PBS (GIBCO-BRL). Recipient mice were killed and DNA extracted from bone marrow, spleen, and thymus for analysis 6 weeks posttransplant.

Southern blot analysis. DNA extracts of murine bone marrow, spleen, and thymus were prepared as described previously.2 For each DNA sample, 5 µg was digested with the restriction enzyme Dra I (Boehringer Mannheim, Indianapolis, IN) and separated by gel electrophoresis in 0.8% agarose (GIBCO-BRL). DNA fragments were transferred onto Zetaprobe nylon membranes (BioRad, Richmond, CA) by capillary transfer overnight in 0.4 N sodium hydroxide. The pY2 probe was labeled using a random prime labeling kit (Boehringer Mannheim), and the blot was probed overnight at 55°C. Autoradiography was performed for varying time intervals. Calculations of percentage male DNA in the samples were adjusted for loading using an interleukin-3 (IL-3) cDNA probe (courtesy of J. Ihle and DNAX, Palo Alto, CA). Percentage engraftment of male cells in the female mice were quantitated using phosphorimage analysis (Molecular Dynamics Inc, Sunnyvale, CA).

FISH. Peripheral blood was taken from transplant recipients by cardiac puncture. Leukocytes were purified using the Easy-Lyse Whole Blood Erythrocyte Lysing kit (Leinco Technologies Inc, Ballwin, MO) and were fixed in Carnoy's solution (75% methanol/25% acetic acid) before being dropped onto microscope slides for FISH analysis. Slides were treated with proteinase K (0.2 µg/mL) (Sigma Chemical Co) in 2 mmol/L Tris buffer, at 37°C and dehydrated through an ethanol series. The DNA was denatured in 70% formamide (GIBCO BRL) at 70°C, slides dehydrated once more, and probe was applied immediately. The probe used in these studies was a digoxigenin-labeled Y chromosome painting probe.18 The probe was incubated on the slides at 37°C overnight. Slides were washed extensively in 50% formamide, 2X SSC followed by 4X SSC and then were stained with rhodamine-antidigoxigenin antibodies (Boehringer Mannheim). Slides were washed with 4X SSC and cells were mounted in antifade solution containing 0.14 mg/mL p-phenylenediamine (Sigma) in 90% glyceter (Fisher Scientific, Pittsburgh, PA) and 0.4 µmol/L DAPI (4,6-Diamidino-2-phenylindole) (Sigma). Positive cells were scored by visual counting against a male and female control. Greater than 98% of every 100 male cells stained positively, and the frequency of false positives was below 1 in 100 female cells (see Pallavicini et al18 for a discussion of FISH sensitivity).

Statistical analysis. Statistical analysis was performed using Wilcoxon's matched pairs test. Each experiment shows an individual mouse for each data point, and within an experiment mice were randomly paired for statistical analysis. The results were considered significant when P < 0.05.

RESULTS

In the present study, we have administered 5FU (150 mg/kg) to BALB/c mice, killed them 6 (FU-6) or 35 (FU-35) days later and compared the capacity of these marrow cells to engraft in host marrow, spleen, or thymus with marrow cells from mice not treated with 5FU. In transplanted female mice analyzed at 10 weeks posttransplantation, there was a significant decrease in engraftment at 6 days post-5FU, confirming previous reports5 (Fig 1 and Table 1). However, in marrow and thymus, engraftment had returned to control values 35 days after 5FU. Recovery of engraftment in splenic tissue appeared incomplete, consistent with our initial observations that marrow and thymic engraftment correlated closely, but splenic engraftment did not correlate well with thymic or marrow engraftment. These data were confirmed in a separate experiment in which engraftment was evaluated 7 weeks after marrow infusion. The results at 7 and 10 weeks posttransplantation were combined and are presented in Table 1 and represent the analysis of 16 to 20 individually transplanted mice. The differences between control and FU-6 or FU-6 and FU-35 marrow recipients for marrow, spleen, and thymus were significant at P values <.02 (FU-6 v normal) and <.05 (FU-6 v FU-35). These data indicate that 5FU administration to donor mice results in a transient engraftment defect into nonirradiated mice that recovers over time.

We have previously shown that engraftment with normal or post-5FU marrow is similar in T cells, B cells, and myeloid cells.3 We have now applied FISH to analyze engraftment levels in the peripheral blood of mice killed 7 weeks after transplantation with normal, FU-6, or FU-35
samples were corrected for loading with an IL-3 probe. Statistical analysis shows that the differences between FU-6 and normal recipients are significant (P < .05).

Southern blots were probed with pY2 and the percentage engraftment was calculated using phosphorimage analysis, taking a male control as 100% and a female control as 0%. All samples were corrected for loading with an IL-3 probe. Statistical analysis shows that the differences between FU-6 and normal recipients are significant (P < .02) and also for FU-6 against FU-35 (P < .05).

Female mice received cells equivalent to two tibiae/two femurs. These results parallel those presented above: 20% ± 3% positive cells in control (646 cells counted from two mice), 7% ± 2% in FU-6 (157 cells counted from two mice) and 18% ± 3% in FU-35 mice (475 cells counted from two mice). This is the first demonstration of engraftment into peripheral blood in unprepared hosts and shows that marrow engraftment is mirrored by the presence of engrafted cells in the peripheral blood.

Post-5FU marrow stem cells are initially induced into active cell cycle at the same time that the above noted engraftment defect occurs. Thus, the cell cycle status of the stem cell may be related to its capacity to engraft and persist in vivo. Accordingly, we have assessed the cell cycle status of the hematopoietic stem cells from normal mice that engraft into nonirradiated hosts. Hydroxyurea selectively kills cells in S phase, and this approach gives essentially identical results to those seen with in vitro high specific activity triti-

![Fig 1. Percentage of male DNA in bone marrow of individual recipient female mice given repetitive transplants of normal, FU-6, or FU-35 bone marrow. Female mice received cells equivalent to two tibiae and two femurs intravenously for 5 days and were killed at 10 weeks posttransplantation. Southern blots were probed with pY2 and the percentage engraftment was calculated using phosphorimage analysis, taking a male control as 100% and a female control as 0%. All samples were corrected for loading with an IL-3 probe. Statistical analysis shows that the differences between FU-6 and normal recipients are significant (P < .02) and also for FU-6 against FU-35 (P < .05).]

5FU pretreatment induces cycling of stem cells assayed in irradiated hosts. If cycle status of stem cells is a determinant of engraftment and explains the poor engraftment seen after 5FU, then it would be anticipated that the majority of engrafting cells in FU-6 marrow would not be cycling; ie, the cycling stem cells will have failed to engraft. We thus used hydroxyurea (or PBS) treatment of FU-6 donors to determine whether the FU-6 marrow cells engrafting into marrow, spleen, or thymus were proliferating or quiescent (Table 3). In these studies, there was a low rate of engraftment with the FU-6 marrow and 73% to 75% of engrafting male cells in female marrow and thymus and 96% of engrafting cells in female spleen were quiescent (not killed by hydroxyurea). These data indicate that of the cells from FU-6 marrow, which engraft in marrow, spleen or thymus, few are proliferating.

**DISCUSSION**

Murine hematopoietic pluripotent renewal stem cells have been defined by their capacity to engraft and repopulate in an irradiated syngeneic mouse. Conventionally, one cell population has been injected at relatively low cell numbers and blood cell repopulation assessed at different times after marrow infusion; in some cases two cell populations have been compared for repopulation in the same irradiated host-

**Table 1. Engraftment of Marrow From 5FU-Treated Mice**

<table>
<thead>
<tr>
<th>Donor Marrow</th>
<th>No. of Recipients</th>
<th>Percent Engraftment ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>16-17</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>FU-6</td>
<td>19-20</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>FU-35</td>
<td>19</td>
<td>34 ± 3</td>
</tr>
</tbody>
</table>

Percentage of male DNA in bone marrow of recipient female mice given repetitive transplants of normal, FU-6, or FU-35 bone marrow. Female mice received cells equivalent to two tibiae and two femurs for 5 days and were killed at 7 or 10 weeks posttransplantation. Southern blots were probed with pY2 and the percentage engraftment was calculated using phosphorimage analysis, taking a male control as 100% and a female control as 0%. All samples were corrected for loading with an IL-3 probe. Statistical analysis showed significant differences between FU-6 and normal (P < .02) and FU-6 and FU-35 (P < .05) for all three tissues.

Abbreviation: SEM, standard error of the mean.
ENGRAFTMENT OF CYCLING MARROW CELLS

Bio Marrow

Table 2. Engraftment of Marrow From Hydroxyurea-Treated Donor Mice

<table>
<thead>
<tr>
<th>Donor Marrow</th>
<th>No. of Recipients</th>
<th>Percent Engraftment ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>10</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>10</td>
<td>57 ± 8</td>
</tr>
</tbody>
</table>

Percentage of male DNA in bone marrow of recipient female mice given repetitive transplants of bone marrow from male mice given 5FU on day 0 and then either PBS or hydroxyurea (900 mg/kg) on day 6, 2 hours before being killed. Female mice received cells equivalent to two tibiae and two femurs from donor males treated with hydroxyurea (900 mg/kg) or saline (controls) and analyzed 12 weeks posttransplantation. Male DNA on Southern blots was detected with the pY2 probe and percentage engraftment was quantitated by phosphorimage analysis. Statistical analysis showed no significant difference between the two groups.

Table 3. Engraftment of Marrow From Donor Mice Treated With 5FU and PBS or 5FU and Hydroxyurea

<table>
<thead>
<tr>
<th>Donor Marrow</th>
<th>No. of Recipients</th>
<th>Percent Engraftment ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5FU + PBS</td>
<td>10</td>
<td>4 ± 0</td>
</tr>
<tr>
<td>5FU + hydroxyurea</td>
<td>10</td>
<td>3 ± 0</td>
</tr>
</tbody>
</table>

Percentage of male DNA in bone marrow of recipient female mice given repetitive transplants of bone marrow from male mice given 5FU on day 0 and then either PBS or hydroxyurea (900 mg/kg) on day 6, 2 hours before being killed. Female mice received cells equivalent to two tibiae and two femurs for 5 days and were killed at 6 weeks posttransplantation. Southern blots were probed with pY2 and the percentage engraftment was calculated using phosphorimage analysis, taking a male control as 100% and a female control as 0%. All samples were corrected for loading with an IL-3 probe. For all tissues analyzed, statistical analysis (Wilcoxon's matched pairs test) showed no significant differences between the two groups.

Previous studies have indicated that marrow procured 6 days after 5FU competes effectively with normal marrow in the irradiated transplant, but is defective in the unprepared host. It has to be noted, however, that these competition assays were performed using C57BL/6 mice and thus may not be comparable to the nonirradiated transplant data in which studies were performed in the BALB/c strain. This difference in engraftment could be because the two models assay intrinsically different stem cells, because stroma is damaged in the irradiated model or because one system monitors a large readout from a few very proliferative cells. Our alternative model assesses the results of competition from relatively large numbers of cells. We are currently comparing various mouse strains and the effects of 5FU-treated donor marrow in both irradiated and nonirradiated hosts. One study from Harrison et al. reports that post-5FU splenic stem cells do not compete effectively with normal splenic stem cells in a long-term repopulation assay in irradiated hosts. These data could be interpreted as damage to the stem cells by 5FU, but could also be consistent with the present results indicating a transient functional engraftment defect after 5FU.

The present data show that the 5FU engraftment defect, which temporally correlates with stem cell proliferation, is transient and not present at 35 days after 5FU. This suggests that the engraftment defect may reflect a relationship between stem cell cycle status and engraftment in this model. Other work from our laboratory has indicated that when marrow cells are exposed to cytokines, they enter cell cycle and lose their ability to engraft in unprepared hosts. This is also consistent with a relationship between stem cell cycle status and engraftment potential. The actual mechanism of engraftment is still unknown. It could be that the engraftment is determined by antigens such as H-Y or other proteins, perhaps integrins, which are modulated by 5FU and/or cell cycle status of the cells.

A competition assay. Repopulation of the irradiated host in the setting of infusion of low numbers of stem cells is not surprisingly, oligoclonal. The irradiated transplanted host represents an amplification system, providing a high yield from small numbers of cells. It also represents a system in which there has been cytotoxic damage to the microenvironmental supporting cells of the bone marrow. This is very different from the model of transplantation into the normal, unprepared host. This model represents a competitive situation in which relatively large numbers of donor marrow cells compete with host marrow. In this model, there is no microenvironmental damage and no significant amplification of cells.

Fig 2. Male cells within the bone marrow of noncytoablated female mice after transplants. Mice were transplanted repetitively for 5 days with cells equivalent to two tibiae and two femurs from donor mice that had received saline. This is consistent with the present results indicating a transient functional engraftment defect after 5FU.

Percentage of male DNA in bone marrow of recipient female mice given repetitive transplants of bone marrow from male mice given 5FU on day 0 and then either PBS or hydroxyurea (900 mg/kg) on day 6, 2 hours before being killed. Female mice received cells equivalent to two tibiae and two femurs for 5 days and were killed at 6 weeks posttransplantation. Southern blots were probed with pY2 and the percentage engraftment was calculated using phosphorimage analysis, taking a male control as 100% and a female control as 0%. All samples were corrected for loading with an IL-3 probe. For all tissues analyzed, statistical analysis (Wilcoxon's matched pairs test) showed no significant differences between the two groups.
5FU is a chemotherapeutic agent that dramatically reduces the number of myeloid and lymphoid cells in normal mice. A major effect of 5FU is to block the enzyme thymididine synthase, which, by methylation, converts deoxyuridine phosphate to deoxythymidine phosphate. When deprived of deoxythymidine phosphate, cells are unable to synthesize DNA and accumulate at the beginning of S phase. Doses of 5FU of 30 and 100 mg/kg cause an irreversible decrease of mitotic and 1HUdR labeling indices. No recovery is seen over 48 hours, showing that there has been cell death.27 Treatment of mice with 5FU results in a marrow population relatively enriched for the primitive in vitro progenitor termed the HPP-CFC.7-9 The most primitive marrow stem cells appear to be dormant and spared by 5FU exposure. Proliferating hematopoietic cells disappear within 2 days28 and, consequently, 5FU treatment induces cycling of primitive engrafting cells.17 The engraftment defect seen after 5FU could, of course, be caused by direct toxic damage to stem cells or an induced differentiation separate from their cycle status. The recovery of the defect over time speaks against, but does not exclude, these possibilities.

If the defect in engraftment seen in marrow cells harvested from mice after pretreatment with 5FU is related to the proliferative status of these cells, then it would follow that cells that do engraft in the nonirradiated host are in a quiescent state. We have assessed the cycle status of these stem cells using selective killing of cells in S phase by hydroxyurea. These data indicate that the normal, unperturbed marrow stem cells, which engraft in unirradiated hosts, are not proliferating. Similarly, the bulk of FU-6 cells, which engraft, are not killed by hydroxyurea. These data, in toto, indicate that actively cycling stem cells do not engraft well in unprepared hosts.

Using irradiated hosts, others have reported cell cycle–related alterations in hematopoietic stem cell phenotype. Early studies on the seeding efficiency of CFU-S in the spleen showed that a number of in vivo perturbations leading to stem cell renewal/proliferation including exposure to vinblastine,39 marrow transplantation30,31 or endotoxin32 led to reduced seeding efficiency. When fetal was compared with adult33,34 or large compared with small colony-forming units,35 the former in each case, shows reduced seeding. One reason for size differences in stem cells relates to position in cell cycle.36-38 Monette and DeMello39 addressed the relationship of proliferative status and engraftment directly comparing seeding efficiency in spleen of colony-forming cells from normal, regenerating and velocity-sedimented cycling and noncycling marrow preparations. Colony forming cells in cycle were found to exhibit a 50% reduction in splenic seeding when compared with normal marrow or sedimented noncycling cells. We have shown that coincident with endotoxin induced proliferation of CFU-S and granulocyte–macrophage colony-forming cell, the transplantation seeding efficiency (f-fraction) of CFU-S was decreased by approximately 30%.40,41 One difficulty with interpreting these studies in irradiated hosts is that CFU-S are a heterogeneous population and, at best, only a percentage of these cells probably represent long-term repopulating cells. More recently, using a highly purified population (Thy-1.1Lin-"Sca-1") of hematopoietic stem cells, Fleming et al5 fractionated Thy-1.1Lin-"Sca-1" on the basis of Hoechst 33342 staining and then compared 100 G0/G1 cells with 100 S/G2/M M cells for radioprotection and donor derived, multilineage reconstitution in peripheral blood. A total of 100 G0/G1 cells protected 90% of lethally irradiated recipients, whereas the 100 S/G2/M cells protected only 22% of recipients. In mice 6 months after reconstitution with G0/G1 cells, 34% of B cells, 10% of myeloid cells, and 19% of T cells were donor. In recipients of S/G2/M cells, only 7.8% B cells and less than 2.0% of both myeloid and T cells were donor derived. Thus, the engraftment of hematopoietic stem cells in the myeloablated host may also be related to cell cycle status of these cells.

We have previously shown that engraftment of either FU-6 or normal marrow in unirradiated hosts was equally reflected in T cells, B cells, and myeloid cells, but did not analyze peripheral blood in these studies.5 Wu and Keating6 have performed studies in untreated recipients indicating that stem cell engraftment may not correlate with cell differentiation. Our studies using FISH indicate, in fact, that engraftment seen in marrow, spleen, and thymus for recipients of normal, FU-6, and FU-35 bone marrow infusions is mirrored by the peripheral blood cells.

Altogether, the present data indicate that the stem cell that engrafts in the nonmyeloblastoid host is a dormant, noncycling cell and suggest that the impact of 5FU on engraftment may relate to inducing dormant stem cells into cell cycle. This type of defect might be missed in the standard irradiated mouse transplant model where a small number of cells can repopulate. These data further suggest major phenotypic changes in stem cells based on cell cycle status. The recovery of engraftment after 5FU suggests that the engraftment defect is not due to a toxic effect or differentiation of the cells. One possibility is that the stem cell differentiation and proliferation phenotype, at least at the more primitive stem cell levels, is dependent on phase of the cell cycle and not determined in a hierarchical fashion.

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