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Improved hematopoiesis in anemic Sl/Sld mice by splenectomy and therapeutic transplantation of a hematopoietic microenvironment

P Anklesaria, TJ FitzGerald, K Kase, A Ohara and JS Greenberger
Improved Hematopoiesis in Anemic SI/SI\(^d\) Mice by Splenectomy and Therapeutic Transplantation of a Hematopoietic Microenvironment

By Pervin Anklesaria, T.J. FitzFgerald, Kenneth Kase, Akira Ohara, and Joel S. Greenberger

The ability of a clonal hematopoiesis-supportive bone-marrow stromal cell line GB1neo\(^d\) to engraft and alter the microenvironment-induced anemia of SI/SI\(^d\) mice was studied. Prior to stromal cell transplantation, SI/SI\(^d\) mice received 1 Gy total body irradiation (TBI) and 13 Gy to the right hind limb. Two months after intravenous (IV) injection of 5 \(\times\) 10\(^8\) GB1neo\(^d\) cells, 54.4\% \pm\ 17.0\% donor origin (G418\(^d\)) colony-forming cells were recovered from the right hind limb of SI/SI\(^d\) mice. Long-term bone marrow cultures (LTBMCs) established from GB1neo\(^d\)-transplanted mice produced 189.5 CFU-GEMM-forming progenitors/flask over 10 weeks compared with 52.7 \(\pm\ 6.2\) CFU-GEMM forming progenitors/flask from irradiated nontransplanted SI/SI\(^d\) mice. A partial correction of macrocytic anemia was observed 2 months after GB1neo\(^d\) transplantation in splenectomized, irradiated SI/SI\(^d\) mice (HgB 7.2 \(\pm\ 0.4\) g/dL; MCV 68.3 \(\pm\ 7.0\) fl) compared to splenectomized, irradiated, nontransplanted SI/SI\(^d\) mice (HgB 6.5 \(\pm\ 1.1\) g/dL; MCV 76.9 \(\pm\ 8.5\) fl) or control SI/SI\(^d\) mice (HgB 5.4 \(\pm\ 0.5\) g/dL; MCV 82.4 \(\pm\ 1.3\) fl). Mean RBC volume distribution analysis showed a 2.5-fold increase in percentage of peripheral blood RBCs with MCV \(\leq\)45 fl and confirmed reduction of the MCV in splenectomized-GB1neo\(^d\)-transplanted mice compared to control SI/SI\(^d\) mice. A hematopoiesis-suppressive clonal stromal cell line derived from LTBMCs of SI/SI\(^d\) mice (SI/neo\(^d\)) engrafted as effectively (43.5\% \pm\ 1.2\% G418\(^d\) CFU-F/limb) as did GB1neo\(^d\) cells (38.3\% \pm\ 0.16\% G418\(^d\) CFU-F/limb) to the irradiated right hind limbs of C57Bl/6 mice. LTBMCs established after 2 or 6 months from SI/neo\(^d\)-transplanted mice showed decreased hematopoiesis (182 \(\pm\ 12\) [2 months] and 3494.3 \(\pm\ 408.1\) [6 months] CFU-GEMM forming progenitors/flask over 10 weeks) compared to those established from GB1neo\(^d\)-transplanted mice (5980 \(\pm\ 530\) [2 months] and 7728 \(\pm\ 607\) [6 months] CFU-GEMM progenitors forming/flask). Thus, transplantation of clonal bone-marrow stromal cell lines in vivo can stably transfer their physiologic properties to normal or mutant mice.

A GENETICALLY determined defect in the bone-marrow microenvironment of SI/SI\(^d\) results in a deficiency of mature erythroid precursors and reduced numbers of pluripotential hematopoietic stem cells. Ultrastructural analysis of the bone-marrow of SI/SI\(^d\) mice shows a reduction in overall cellularity. SI/SI\(^d\) mice are not cured by marrow stem cells from normal donors. Long-term bone marrow cultures (LTBMCs) established from SI/SI\(^d\) mouse show decreased cumulative production of hematopoietic progenitor cells compared with normal littermate mice. SI/SI\(^d\) mice has been shown to restore some hematopoietic support capacity. Recent studies with permanent clonal stromal cell lines derived from LTBMCs of SI/SI\(^d\) mice demonstrate that some lines suppress hematopoiesis in vivo. The available evidence supports the conclusion that abnormal hematopoiesis observed in SI/SI\(^d\) mice is due to a defect in cells of the hematopoietic microenvironment.

Several types of transplantation experiments have been attempted as therapy for the SI/SI\(^d\) anemia, including parabiosis, transplantation of whole spleens, and implantation of normal mouse femurs into the abdominal wall. An increase in total cellularity and hematopoietic progenitors was reported in one of these studies but with no significant improvement in clinical macrocytic anemia. In the present studies we used a technique for bone-marrow stromal cell transplantation that involves total body irradiation (TBI) and boost irradiation to an isolated extremity followed by intravenous (IV) injection of a clonal-marrow stromal cell line. The clonal bone-marrow stromal cell line GB1/6 derived from LTBMCs has been shown to support hematopoietic stem cells in vitro, engrafts to targeted marrow sites, and supports hematopoietic recovery in vivo in sublethally irradiated normal mice. We studied the effects of transplantation of GB1/6 stromal cell line into SI/SI\(^d\) recipient mice and in a reciprocal experiment the effect of transplanting a hematopoiesis-suppressive clonal stromal cell line (SI/3\(^d\)) derived from the SI/SI\(^d\) mouse marrow microenvironment into normal mice. The data provide evidence for physiologic long-term stability and thus the therapeutic potential of marrow stromal-cell transplantation.

MATERIALS AND METHODS

Mice. Adult, 5- to 8-week-old, severely anemic WCB6F1 SI/SI\(^d\) mice and C57Bl/6 mice were obtained from Jackson Laboratories, Bar Harbor, ME. B6\(^d\)Cast\(^*\)-GPI\(^-\)a\(^-\) mice, obtained from Jackson Laboratories, were bred at the University of Massachusetts Medical Center.

Bone-marrow stromal cell lines. Derivation and characterization of clonal-marrow stromal cell lines (GB1/6, GB1neo\(^d\), SI/3\(^d\), +/+ 2.4 and D2XR11) have been described previously. The embryo fibroblast cell line BI/6embC was provided by Dr S. Aaronson, NCI, Bethesda, MD. Neomycin (G418\(^d\))-resistant subclones of SI/3\(^d\) and D2XR11 and BI/6embC were established by retroviral vector-mediated gene transfer. The subclones are designated SI/3\(^d\)neo\(^d\), D2Xneo\(^d\), and BI/6embneo\(^d\), respectively.

Total body and hind-limb boost irradiation. Adult recipient SI/SI\(^d\) or C57Bl/6 mice received 1 to 2 Gy TBI and 10.0 to 20.0 Gy
to the right hind limb (RHL) or both hind limbs (BHL) delivered by a linear accelerator as described.14 We used 1 Gy TBI and 10 Gy to the RHL due to the relative sensitivity of SI/SI6 mice to TBI.2 Irradiated mice were transplanted with 5 x 10^6 stromal cells of each line (GBln eo, SI/S neo, Bl6/emb neo C or D2X neo) by IV injection (single schedule). For sequential boost irradiation-transplantation studies with SI/SI6 mice, 1 Gy TBI and 10 Gy to the RHL was delivered by linear accelerator on day 0. A single-cell suspension of the GBlneo cell line was injected IV 48 hours later. Two months after the first irradiation schedule, the same group of mice received 1 Gy TBI and 10 Gy to the left hind limb (LHL). Another injection of the GBlneo cell line was administered 48 hours later (multiple transplant schedule). Control irradiated nontransplanted mice received 2 Gy TBI and 10 Gy to both hind limbs (BHL).

Splenectomy and irradiation of SI/SI6 mice. SI/SI6 mice (5 to 6 weeks old) were anesthetized using an inhalation anesthetic me-thoxyflurane (metofane, Pitman Moore, Inc, Washington Crossing, NJ). A small incision was made in the flank region, and the spleen was gently removed and cauterized as described.14 The incision was sutured with a 5.0 chromic gut and the skin flaps held together with wound clips using Mikron auto clip (Clay Adams). Two weeks after splenectomy, mice were irradiated (1 Gy TBI and 10 Gy BHL) and transplanted (single schedule) with the GBlneo marrow stromal cell line as described above. Transplanted and control animals were followed for clinical improvement of their sampled blood Hgb concentration, hematocrit, and mean corpuscular volume (MCV), as described below.

Measurement of stromal cell engraftment. The CFU-F assay was carried out as described.14 Briefly, control-irradiated–nontransplanted or irradiated-transplanted mice were sacrificed at time intervals indicated, and the harvested bone-marrow cells from each hind limb were counted and plated at different cell concentrations in 60-mm dishes (Falcon, Lincoln Park, NJ) in the absence or presence of 200 μg/mL G418 (Gibco). CFU-F colonies (>25 cells) were counted at days 7 and 14.

Measurement of hematopoietic recovery in vivo and in vitro. Peripheral blood from transplanted and control-irradiated–nontransplanted mice was analyzed monthly. Blood from the tail vein (80μL) was collected in heparinized microhematocrit capillary tubes (Fisher Scientific, Springfield, NJ) and mixed with 20 mL of diluent (NaCl [6.38 g/L], Boric acid [1.0 g/L], sodium tetraborate [0.2 g/L], and EDTA-2K [0.2 g/L]). RBCs, WBCs, platelets, Hgb concentration, hematocrit, and MCV were measured using an automated TOA-II Sysmex (American Scientific Products, Stone Mountain, GA). Mean volume distribution studies of RBCs obtained from control-irradiated–splenectomized and transplanted mice were studied using a Coulter Counter with a Channalizer (Coulter, Hialeah, FL). Peripheral blood smears were made and stained with Wright's-Giemsa stain.

The functional integrity of transplanted stromal cells was evaluated by establishing LTBMCs from each hind limb as described.15 Hematopoietic recovery was quantitated by measuring cumulative nonadherent CFU-GE/MM–forming progenitor cells removed weekly for over 10 weeks in culture.16

In vitro hematopoietic progenitor cell engraftment to clonal stromal cell lines. Plateau-phase cultures of each stromal cell line (GBln eo or SI/S neo) were individually established by plating cells in 25 cm² flasks (Corning Plastics, New York). The confluent stromal cell cultures were engrafted with 2.5 to 3.0 x 10⁶ washed, nonadherent hematopoietic progenitor cells harvested from 4- to 6-week-old LTBMCs established from C57Bl/6 mice.9 At weekly intervals after engraftment, nonadherent cells were harvested and assayed for CFU-GE/MM–forming progenitor cells. Colony assays were performed using pokeweed-mitogen–stimulated spleen cell-conditioned medium (PWM-SCCM) and erythropoietin (EPO) as described.14

In other experiments, different ratios of the two stromal cell lines were added to same flasks to test for dominance of biologic properties.

Statistical evaluation. The differences between the means was determined by standard Student’s test and chi-square analysis.9

RESULTS

Recovery of bone-marrow stromal cells in vitro after TBI and isolated hind-limb boost irradiation in vivo. The effects of TBI (1 to 2 Gy) and high-dose irradiation to the RHL (13 Gy) on regeneration of normal C57Bl/6 and SI/SI6 mouse-marrow stromal cells in vivo were studied first. Two days following 1 Gy TBI and 13 Gy to the RHL, the femoral CFU-F from normal C57Bl/6 mice decreased to 53% of the level detected in control-nonirradiated mice (Fig 1). By 60 days after irradiation, a further decrease (26% of control) in the colony-forming potential of marrow stromal cells from irradiated C57Bl/6 mice was observed (P < .05). Improvement in the colony-forming ability of stromal cells from irradiated mice at 180 days after irradiation was not significant (Fig 1).

The number of colony-forming CFU-F progenitors/hind limb obtained from nonirradiated SI/SI6 mice was 53% lower than that of control nonirradiated C57Bl/6 mice. At two
days after irradiation, the number of CFU-F colonies obtained from irradiated SI/SI\(^4\) mice was not significantly lower than that of control-nonirradiated SI/SI\(^4\) mice. However, by 60 days after irradiation, there was a 41% decrease in the colony-forming ability of stromal cells from irradiated SI/SI\(^4\) mice (P < .05, Fig 1). These data indicated that the schedule of TBI and boost irradiation would reduce the number of colony-forming host stromal cells and should provide space to support the seeding of engrafted stromal cells at this site.

Support of hematopoietic progenitor cells by stromal cell cultures derived from different ratios of SI\(^4\)neo' and GBlneo'. To estimate the number of GBlneo' cells required to overcome the suppressive marrow microenvironment of SI/SI\(^4\) mice, we first derived plateau-phase stromal cultures in vitro from different ratios of SI\(^4\)neo' and GBlneo' cells. These mixed stromal-cell cultures were then engrafted with day 40 hematopoietic progenitor cells from LTBMCs of Bl/6 mice. As shown in Table 1, cultures of 100% GBlneo' cells supported hematopoiesis for over 4 weeks. In contrast, cultures of 100% SI\(^4\)neo' cell line supported 1% of the number of progenitor cells compared to the GBlneo' cell cultures (P < .05). Plateau-phase cultures with equal numbers of GBlneo' and SI\(^4\)neo' cells (1:1) supported less than 2% of the number of hematopoietic progenitors. The number of progenitors supported by stromal cultures at a 1:10 ratio of GBlneo':SI\(^4\)neo' was 2% of the level supported by cultures of GBlneo' cells (P < .05). Increasing the numbers of GBlneo' cells (tenfold, 50-fold, or 100-fold) resulted in a 17% increase in the number of CFU-GEMM-forming progenitors supported compared to the cultures containing equal numbers of GBlneo' and SI\(^4\)neo' cells (Table 1). If the results with mixed cultures in vitro were extrapolated to predict the results of engrafting new stromal cells in vivo, then up to 80% of the post-transplant hematopoietic microenvironment of SI/SI\(^4\) mice might have to be replaced by GBlneo' cells to observe a detectable biological effect.

Stromal cell line transplantation in vivo improves hematopoiesis in splenectomized SI/SI\(^4\) mice. The ability of GBlneo' cells to stably engraft into irradiated SI/SI\(^4\) mice was first evaluated. Explanted marrow cells from control-nontransplanted mice and GBlneo'-transplanted mice were selected in 200 μg/mL G418. Two months after transplantation, 54.4% ± 17% of the stromal cell colonies obtained from SI/SI\(^4\) mice transplanted with GBlneo' cell line were G418\(^+\) and of donor origin (Table 2, group 2). Control-irradiated–nontransplanted SI/SI\(^4\) mice showed no detectable G418\(^+\) adherent CFU-F (Table 2, group 4b). In comparison, the number of GBlneo' CFU-F recovered from irradiated transplanted Bl/6 mice at 2 months was 38.3% ± 0.16% of the total recovered from the RHL (Table 2, group 1). Thus the GBlneo' cell line engrafted with equal efficiency to SI/SI\(^4\) or C57Bl/6 mice. At 6 months after transplant, no donor origin G418\(^+\) CFU-F were detected in the RHL of SI/SI\(^4\) recipients.

### Table 1. Support of Hematopoietic Progenitors by Stromal Cell Cultures Derived From Different Ratios of SI\(^4\)neo' and GBlneo' Clonal Stromal Cell Lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Ratio Plated*</th>
<th>Cumulative CFU-GEMM Progenitors/Flask †</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI(^4)neo'</td>
<td>1:10</td>
<td>21.5 ± 2.5</td>
</tr>
<tr>
<td>GBlneo'</td>
<td>1:10</td>
<td>1867 ± 218</td>
</tr>
<tr>
<td>SI(^4)neo', GBlneo'</td>
<td>1:10</td>
<td>33 ± 1.8</td>
</tr>
<tr>
<td>SI(^4)neo', GBlneo'</td>
<td>1:50</td>
<td>584.5 ± 85.5</td>
</tr>
<tr>
<td>SI(^4)neo', GBlneo'</td>
<td>1:100</td>
<td>450 ± 79.6</td>
</tr>
<tr>
<td>SI(^4)neo', GBlneo'</td>
<td>10:1</td>
<td>33.6 ± 6.7</td>
</tr>
</tbody>
</table>

*Stromal cells plated at different ratios in 25-cm\(^2\) flasks in duplicate to form confluent cultures with no further cell division possible, thus maintaining a stable ratio. Day 36 nonadherent hematopoietic cells (3 × 10\(^6\) cells) from C57BL/6 LTBMCs were then engrafted to each flask. At weekly intervals, nonadherent cells produced/flask were harvested and assayed for CFU-GEMM-forming progenitors.

†Results are expressed as mean ± SD day 34 cumulative CFU-GEMM-forming progenitors/flask from two separate experiments.

### Table 2. Recovery of Donor Origin Cells From Hind Limb Explants of Mice Transplanted With Clonal Stromal Cell Lines

<table>
<thead>
<tr>
<th>Group*</th>
<th>Total No. of Stromal Cell Colonies Per Hind Limb</th>
<th>Total No. of G418-Resistant Donor Origin Stromal Cell Colonies Per Hind Limb †</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>1. C57Bl/6 mice transplanted with GBlneo' 8.1 cells</td>
<td>78.0 ± 13.0</td>
<td>98.7 ± 13.3</td>
</tr>
<tr>
<td>2. SI/SI(^4) mice transplanted with GBlneo' 8.1 cells</td>
<td>182.4 ± 9.1</td>
<td>126.0 ± 47.0</td>
</tr>
<tr>
<td>3. C57Bl/6 mice cells transplanted with SI(^4)neo' 3.3 cells</td>
<td>88.5 ± 7.0</td>
<td>90.0 ± 20.3</td>
</tr>
<tr>
<td>4. Control-irradiated–nontransplanted (a) (C57Bl/6 mice) or (b) (SI/SI(^4) mice)</td>
<td>65.5 ± 4.1</td>
<td>110.1 ± 10.3</td>
</tr>
</tbody>
</table>

Abbreviation: NT, not tested.

*The C57Bl/6 mice received 3 Gy TBI and 13 Gy to the RHL; SI/SI\(^4\) mice received 1 Gy TBI and 11 Gy to the RHL. Results are expressed as mean ± SD for three to five animals/group.

†The values in parenthesis are % of the values obtained in cultures without G418.
C57BI/6 mice (Table 2, group I).

CFU-F colonies were still detected in GBlneor transplanted sequential boost counts 60 days showed no significant Si/Si" at 2 months, compared with nonirradiated recovery in vivo at 120 days (Table 2, group IVb).

In contrast, at 6 months, 22.5% G41 8 suppressive microenvironment of the mice transplanted with GBlneor cells (single schedule) showed no significant hematopoietic recovery in vivo at 120 days (Table 2, group IVb).

To determine whether the failure to achieve detectable hematopoietic recovery in the GBlneor-transplanted mice was attributable to the inability of these cells to overcome the suppressive microenvironment of the Si/Si" anemia, we next repeated the transplant studies in splenectomized Si/Si" mice. A group of Si/Si" mice were splenectomized 15 days before TBI and hind-limb irradiation (10 Gy BHL) and then transplanted with the GBlneor cell line. Splenectomized, irradiated, GBlneor-transplanted mice had significantly increased WBC counts and Hgb concentration (9.5 ± 1.4 x 10^3/µL and 72. ± 0.4 g/dL, respectively [Table 3, group V]) at 2 months, compared with nonirradiated Si/Si" mice (WBC counts: 4.6 ± 0.6 x 10^3/µL; Hgb concentration: 5.4 ± 0.5 g/dL, Table 3, group III P < .05). The splenectomized GBlneor-transplanted mice at 2 months had reduced MCV (68.3 ± 7.0 FL) compared with that from control mice (82.4 ± 3 FL; P < .05, Table 3, group II). RBC volume distribution analysis demonstrated clear differences in RBC volumes comparing SI/SI" mice to normal C57B1/6 mice, with only a small overlap between the two populations (Fig 2). The median MCV for normal RBCs from C57B1/6 mice was 30 FL (range 16 to 45 FL) and that for SI/SI" mice was 78 FL (range 52 to 83 FL; Fig 2). In the peripheral blood RBC population obtained from splenectomized GBlneor-transplanted SI/SI" mice, there was a 2.5- and 1.5-fold increase in the percentage of cells with MCV of 45 FL and 52 FL respectively as compared with those obtained from SI/SI" mice (Fig 2). This shift in red cell volume to normal levels suggests a detectable partial correction of the macrocytic anemia in vivo in GBlneor-transplanted mice.

The physiologic function of GBlneor cells in different groups of transplanted SI/SI" mice was next evaluated by in vitro culture of marrow. The first group consisted of mice receiving 1 Gy TBI and 13 Gy to the RHL. Marrow cultures established from irradiated-nontransplanted SI/SI" mice showed increased cumulative progenitor cell production per flask (RHL, 10 Gy; 52 ± 10 progenitors/flask) compared to those from nonirradiated control mice (16 ± 4 progenitors/flask; P < .01; Fig 3). The cumulative number of nonadherent CFU-GEMM-forming progenitors produced in LTBMCs from RHL (10 Gy) of GBlneor-transplanted SI/SI" mice was 189.5 progenitors/flask. This was higher than that produced either in LTBMCs from control-nonirradiated or control-irradiated-nontransplanted mice. In contrast, LTBMCs established from the left hind limb of transplanted mice (1 Gy) supported multipotential progenitor cell production less efficiently (46.5 CFU-GEMM forming progenitors/flask; Fig 3). In the next two groups tested, we attempted to replace a dominant fraction of the marrow microenvironment of SI/SI" mice by preparing the mice with either 2 Gy TBI and 20 Gy to both hind limbs (BHL, single schedule) or by sequential irradiation transplantation (multiple schedule), as described in Materials and Methods. In both experiments none of the control-irradiated–nontransplanted mice survived. LTBMCs established from GBlneor-transplanted mice (2 Gy TBI and 20 Gy BHL) at 4 months showed increased cumulative FU-CFU-GEMM-forming progenitors/flask in both right (298.8 ± 32.7) and left (415.8 ± 36.5) hind limb cultures compared to nonirradiated SI/SI" mice (P < .05; Fig 4). In separate experiments, 2 months after mice received the second irradiation transplantation, the cumulative number of CFU-GEMM-forming progenitors obtained per flask was higher in LTBMCs established from engrafted RHL (136.1 ± 32) and LHL (78.6 ± 15.4) of GBlneor-transplanted mice compared with those from control-nonirradiated SI/SI" mice (P < .05; Fig

<table>
<thead>
<tr>
<th>Group*</th>
<th>Peripheral Blood Analysis</th>
<th>Peripheral Blood Analysis</th>
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<tbody>
<tr>
<td></td>
<td>WBC x 10^3/µL</td>
<td>RBC x 10^3/µL</td>
</tr>
<tr>
<td>I. Normal C57B1/6</td>
<td>7.5 ± 1.7</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td>II. Nonirradiated SI/SI&quot;</td>
<td>4.6 ± 0.67</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>III. Irradiated-nontransplanted SI/SI&quot;</td>
<td>4.5 ± 0.7</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>IV. SI/SI&quot; transplanted with GBlneor</td>
<td>4.5 ± 0.7</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>a) At 60 days (RHL)</td>
<td>4.3 ± 0.5</td>
<td>2.57 ± 0.37</td>
</tr>
<tr>
<td>b) At 60 days (BHL)</td>
<td>3.26 ± 0.5</td>
<td>1.9 ± 0.57</td>
</tr>
<tr>
<td>V. SI/SI&quot; splenectomized and transplanted with GBlneor (BHL)</td>
<td>9.5 ± 1.4‡</td>
<td>3.15 ± 0.2</td>
</tr>
<tr>
<td>VI. SI/SI&quot; splenectomized irradiated nontransplanted</td>
<td>7.6 ± 1.9</td>
<td>2.24 ± 0.6</td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± SD for at least three to five mice per group.
†In group (b) mice received sequential irradiation-transplantation (multiple schedule), and all control-irradiated–nontransplanted mice died within 15 days after second irradiation. 
‡P < .05 as compared to control-nontransplanted SI/SI" mice.
Thus the transplanted GBlneo' cells functioned both in vivo in splenectomized SI/SI' mice to support hematopoiesis and in vitro after explant to LTBMC.

Transplantation of the S1'neo' stromal cell line to irradiated C57BL/6 mice. Normal C57BL/6 mice that received TBI and RHL irradiation (1 Gy TBI and 10 Gy RHL) were transplanted with the S1'neo' stromal cell line. At 2 and 6 months after transplantation, adherent stromal cells explanted from femurs and tibias of transplanted mice were selected in G418, and the percentage of donor-origin stromal cells recovered was 43.5% ± 1.2% and 8% ± 4.5% respectively (Table 2, group 3). Thus the S1'neo' cell line engrafted in vivo to Bl/6 mice as efficiently as the Gbneo' cell line (Table 2, group 1). Marrow cultures established from mice transplanted with the Gbneo' stromal cell line had significantly higher CFU-GEMM-forming progenitors per flask (2 months 5980 ± 530; 6 months 7728 ± 607, P < .05) compared with those from either control-irradiated–nontransplanted or Sineo' transplanted mice (Fig 5). In contrast, the cumulative number of hematopoietic progenitors supported by LTBMCs established at 2 and 6 months post-transplantation from S1'neo' transplanted mice was 182 ± 12 and 3494 ± 408 CFU-GEMM progenitors/flask, respectively, and was no higher than that detected with marrow cultures...
STROMAL CELL TRANSPLANT

Fig 4. Support of hematopoietic progenitors harvested from LTBMCs established from SI/SI° mice after 2 Gy TBI and 20 Gy to BHL (single schedule) or 1 Gy TBI and 10 Gy RHL followed by a second irradiation dose of 1 Gy TBI and 10 Gy LHL 2 months later (multiple transplant schedule). At 4 months after irradiation-transplantation, LTBMCs were established from three mice/group. Control, nonirradiated SI/SI° mice (—O—), GBlneo’ transplanted RHL-20 Gy (—□—), LHL-20 Gy (—■—), sequentially irradiated GBlneo’-transplanted RHL-10 Gy (—■—), and LHL-10 Gy (—□—). Results are expressed as mean ± SD of cumulative, nonadherent CFU-GEMM progenitors/flask.

established from control-irradiated—nontransplanted mice (219 ± 9 [2 months] and 3225 ± 363 [6 months] CFU-GEMM—forming progenitors/flask; Fig 5). Thus both the GBlneo’ and SI°neo’ cells transferred their in vitro biologic properties to the in vivo marrow microenvironment of C57Bl/6 mice.

DISCUSSION

The present studies demonstrate that the GBlneo’ marrow stromal cell line engrafts in vivo and improves hematopoietic recovery in splenectomized SI/SI° mice. Another stromal cell line, SI°neo’, does not support hematopoiesis in vitro but engrafts in vivo and transfers its biologic properties to irradiated C57Bl/6 mice. Thus distinct physiologic properties of two clonal stromal cell lines that are expressed in vitro are maintained following transplantation in vivo.

The engrafted GBlneo’ cells in splenectomized irradiated SI/SI° mice induced an increase in the HgB concentration as well as WBC counts in the peripheral blood of these mice as compared to that of control or splenectomized mice. The present data confirm and extend a previous report that demonstrated a partial stimulation of erythropoiesis in splenectomized SI/SI° mice implanted with intact spleens from +/+ mice. A decrease in the average MCV of peripheral

Fig 5. Hematopoietic progenitors produced in LTBMCs established from the RHLs of SI°neo’ and GBlneo’ transplanted C57BL/6 mice. At 2 and 6 months after transplantation, three to five mice per group were sacrificed and LTBMCs established from RHL. Results are expressed as mean ± SD cumulative, nonadherent CFU-GEMM progenitors/flask from control-irradiated—nontransplanted mice at 2 (—■—) and 6 (—□—) months, from GBlneo’-transplanted mice at 2 (—■—) and 6 (—□—) months; and from SI°neo’-transplanted mice at 2 (—□—) and 6 (—■—) months.
blood RBCs was also observed in splenectomized GBlneo' transplanted mice. Furthermore, LTBMCS established at 2 and 6 months from GBlneo'-transplanted SI/SI<sup>a</sup> mice showed increased longevity compared to marrow cultures from control SI/SI<sup>a</sup> mice. This may be attributable to a growth advantage in vitro of GBlneo' stromal cells that further increase the favorable ratio of supportive stromal cells in vitro. LTBMCS established from control-irradiated SI/SI<sup>a</sup> mice supported hematopoiesis more efficiently than those from nonirradiated-control SI/SI<sup>a</sup> mice. Thus in SI/SI<sup>a</sup> mice, high-dose irradiation may help create a "niche" in the marrow cavity to support transplanted stromal cells and may also eliminate endogenous stromal cells that suppress hematopoiesis.

It is not yet known what characteristics are important for stable engraftment of stromal cell lines in vivo. A clonal murine embryo fibroblast (Bl/6embneo' c) and bone-marrow stromal cell line (GBlneo') that support hematopoiesis in vitro can engraft stably in vivo for up to 12 months to irradiated marrow sinuses of normal mice (unpublished observations). However, GBlneo' cells were not detected by the CFU-F assay in the bone marrow of mutant SI/SI<sup>a</sup> mice at 6 or 12 months. One possible explanation for these data may be that the number of donor-origin cells was below the level of sensitivity of the assay used (G418<sup>+</sup>) for detecting donor-origin cells. Another possibility could be that either migration or regeneration of endogenous SI/SI<sup>a</sup> stromal cells in irradiated marrow sinuses may have eliminated or reduced the numbers GBlneo' cells. Results obtained in vitro using stromal cultures made up of different ratios of SI<sup>a</sup>neo' and GBlneo' cells indicated that one tenth the number of SI<sup>a</sup>neo' cells could suppress support of hematopoiesis by GBlneo' cells. While the SI<sup>a</sup>neo' stromal cell line did not support hematopoiesis in vitro, it did engraft in vivo and stably transferred its suppressive microenvironment. The stromal cell line D2Xneo', which does support hematopoiesis in vitro, did not engraft in vivo (unpublished observations). Thus the parameter of in vivo engraftment of a stromal cell line does not necessarily correlate with hematopoietic support in vitro and appears to be an independent biologic marker.

The present results indicate improved hematopoiesis in anemic SI/SI<sup>a</sup> mice after splenectomy and engraftment of a clonal bone-marrow stromal cell line. These data support the theory that the splenic microenvironment may be involved in the pathogenesis of some types of chronic anemia.23-27 Our attempts to replace a dominant fraction of the hematopoietic microenvironment involved sequential high-dose irradiation of two marrow niches and removal of the abnormal spleen microenvironment by splenectomy. While the parameters of stromal-cell transplantation need to be optimized, the present results provide evidence that therapeutic stromal cell transplant may be a viable alternative in diseases associated with a defective marrow microenvironment.20-27

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