Characterization of Peripheral Blood Stem Cells in Mice

By Yoshihisa Yamamoto, Ryoji Yasumizu, Yasuo Amou, Naoyo Watanabe, Nobuhiro Nishio, Junko Toki, Shirou Fukuhara, and Susumu Ikehara

Peripheral blood stem cells (PBSCs) were mobilized in mice by treatment with cytosine-arabinoside on day 0, followed by the administration by injection of granulocyte colony-stimulating factor for 4 days. There were remarkable increases in the numbers of cells with lineage-negative (Lin−) c-kit+ markers, cells with colony-forming unit-cell (CFU-C) and colony-forming unit-spleen (CFU-S) activities, and cells with marrow-repopulating ability (MRA) in the extramedullary sites (the spleen, peripheral blood, and liver) on day 5, whereas the number of these immature hematopoietic cells decreased in the bone marrow (BM) on day 5. This finding suggests the mobilization of immature hematopoietic cells from the BM to the extramedullary sites. Three-color flow cytometric analyses showed that CD4 antigen was not expressed on the Lin−Sca-1+ cells in the mobilized PB cells.

PBSCs can provide long-term (24 weeks) multilineage hematopoiesis in allogeneic mice across the major histocompatibility complex barrier without severe graft-versus-host disease (GVHD). To answer this question, we examined changes in the distribution and activity of immature hematopoietic cells after the mobilizing treatment, whereas the number of these immature hematopoietic cells decreased in the bone marrow (BM) on day 5. This finding suggests the mobilization of immature hematopoietic cells from the BM to the extramedullary sites. Three-color flow cytometric analyses showed that CD4 antigen was not expressed on the Lin−Sca-1+ cells in the mobilized PB cells.

Puripotent hematopoietic stem cells (P-HSCs) have two characteristics: the capacity for eternal self-renewal and the ability to differentiate into all cell lineages, including lymphoid cells. The differentiation of P-HSCs into committed progenitor cells is continuous and results in a hierarchical structure.1,2

Recently, there has been much interest in HSCs that are mobilized into the peripheral blood (PB) after chemotherapy and/or stimulation with cytokines.1,4 However, it should be clarified as to whether PB stem cells (PBSCs) are the same P-HSCs as those present in the bone marrow (BM), because PBSCs are now used for gene therapy and allogeneic transplantation.4,10 To answer this question, we examined changes in the distribution and activity of immature hematopoietic cells after mobilization in mice with or without splenectomy. Splenectomy was performed because (1) some PBSCs may be mobilized from the spleen and (2) there may be changes in the distribution of mobilized cells after splenectomy, because it is conceivable that mobilized cells migrate into the spleen.

We show here that there are remarkable increases in the numbers of cells with lineage-negative (Lin−) c-kit+ markers, cells with colony-forming unit-cell (CFU-C) and colony-forming unit-spleen (CFU-S) activities, and cells with marrow-repopulating ability (MRA) in the extramedullary sites after the mobilizing treatment, whereas the number of these immature hematopoietic cells decreased in the BM; also, the PBSCs express different antigens from HSCs in the BM. We also show that the transplantation of T-cell-depleted PBSCs can provide long-term (24 weeks) multilineage hematopoiesis in allogeneic mice across the major histocompatibility complex (MHC) barrier without severe graft-versus-host disease (GVHD).

MATERIALS AND METHODS

Mice. C57BL/6J (B6) and C3H/HeN (C3H) mice were purchased from Japan CLEA Inc (Osaka, Japan) and maintained in our animal facility. These mice were used between 8 and 12 weeks of age. Some B6 mice were splenectomized at 4 weeks of age to examine the change in the distribution of hematopoietic cells after cytosine-arabinoside (AraC) plus granulocyte colony-stimulating factor (G-CSF) treatment.

Hematopoietic growth factors. Recombinant murine interleukin-3 (rniL-3), recombinant murine stem cell factor (rmSCF), recombinant human G-CSF (rhG-CSF), rhIL-6, and recombinant human erythropoietin (rhEpo) were kindly provided by Kirin Brewery Co, Ltd (Tokyo, Japan). The standard concentrations of the hematopoietic growth factors used in our culture were as follows: 100 ng/mL SCF, 100 U/mL IL-3, 100 ng/mL IL-6, 100 ng/mL G-CSF, and 2 U/mL Epo.

Protocol of PBSC mobilization. Three to eight mice were injected intraperitoneally with AraC (200 mg/kg; Nippon Shinyaku Co, Ltd, Kyoto, Japan) twice at a 6-hour interval on day 0, followed by a subcutaneous injection of 250 μg/kg of G-CSF on days 1 through 4. AraC was administered by injection at 10:00 am and 4:00 pm. G-CSF was diluted in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) before subcutaneous injection. Injections were administered at 9:00 am and 9:00 pm, and mice were killed 12 hours after the last injection.

Isolation of hematopoietic cells. PB was harvested from the orbital plexus under anesthesia. Approximately 1 mL of blood was collected per mouse and centrifuged over Lympholyte-M (density, 1.0875; Cedarlane, Homby, Ontario, Canada) to remove erythrocytes. All nucleated cells from the interface were collected, washed once with PBS containing 2% fetal calf serum (2% FCS-PBS), and resuspended in 2% FCS-PBS.

BM cells (BMCs) from the femurs and tibias were collected by flushing. Spleens were removed and mechanically dissociated. The single-cell suspensions were obtained by passing through a 21-gauge needle. Cells were washed once with cold 2% FCS-PBS and resuspended in 2% FCS-PBS.

Liver mononuclear cells were obtained as follows. The liver was slowly perfused by 10 mL of warm (37°C) PBS containing 100 U/
ml of collagenase type IV (Sigma, St Louis, MO) to remove PB and digest the tissue. The liver was then excised, minced, and centrifuged over Lympholyte-M, and the mononuclear cells were then treated as described above.

**Hematopoietic progenitor assays.** The CFU-C assay was performed by culturing cells in 1 ml of 0.8% methylcellulose, 30% FCS, 1% BSA, 5 x 10^{-3} mol/L 2-mercaptopethanol (2-ME), 100 U/ml IL-3, and 2 U/ml Epo. The colonies consisting of 50 or more cells were scored under an inverted microscope after 7 days of culture. In some experiments, the cells were plated in designated hematopoietic growth factors and scored after 7 days on the basis of gross morphology as granulocyte-macrophage (colony-forming unit–granulocyte-macrophage [CFU-GM]), erythroid burst (burst-forming unit-erythroid [BFU-E]), or mix (colony-forming unit granulocyte, erythroid, monocyte, megakaryocyte [CFU-GEMM]).

The CFU-S assay was performed by injecting limited numbers of test cells into the orbital cavity of four to eight lethally irradiated mice (850 cGy at 90 cGy/min). The spleens were removed 8 or 12 days later and fixed with Bouin's solution. Visible surface colonies were counted and scored as CFU-S. Control mice (irradiation only) showed no macroscopic spleen nodules on days 8 or 12.

The MRA was investigated using a method described previously. Briefly, 13 days after the injection of test cells into lethally irradiated mice, aliquots of their femurs were used for the CFU-C assays. The MRA was expressed as CFU-C colonies generated per femur of host mice per 10^5 test cells injected.

**Monoclonal antibodies (MoAbs) and labeling reagent.** The following MoAbs were used for cell surface analyses and cell sorting: anti-B220 (RA3-6B2), anti-Mac-1 (M1170.15), anti-Gr-1 (RB6-8C5), anti-CD4 (YTS191.1 or RM4-4), anti-LFA-1 (M27/5.2), anti-CD71 (C2), anti-VLA-4 (R1-2), anti-LFA-1 (M27/5.2), and anti--Pgp-1 (1M7) MoAbs. Most MoAbs were obtained as purified anti-CD4 or reagents for 30 minutes on ice. Staining of cells with Rh123 was followed by postosmification. After dehydration with graded ethanol, the sorted cells were fixed with 2% glutaraldehyde containing 0.1 mom phosphate buffer (pH 7.4), followed by postosmification. After dehydration with graded ethanol, they were embedded in Epon (Oken Commercial Co, Ltd, Tokyo, Japan). Ultrathin sections prepared by a Porter-Blum ultramicrotome (Sorvall Inc, Newtown, CT) were observed using a Hitachi H-600 electron microscope (Hitachi, Ibaragi, Japan) after double staining with uranyl acetate and lead citrate.

**Electron microscopic study.** The sorted cells were fixed with 2% glutaraldehyde containing 0.1 mol/L phosphate buffer (pH 7.4), followed by postosmification. After dehydration with graded ethanol, they were embedded in Epon (Oken Commercial Co, Ltd, Tokyo, Japan). Ultrathin sections prepared by a Porter-Blum ultramicrotome (Sorvall Inc, Newtown, CT) were observed using a Hitachi H-600 electron microscope (Hitachi, Ibaragi, Japan) after double staining with uranyl acetate and lead citrate.

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Each experiment was performed three or more times. Reproducible results were obtained, and representative data are, therefore, shown in the tables and figures.

**RESULTS**

**Time course in response to AraC and G-CSF.** Three to eight nonsplenectomized or splenectomized C57BL/6 mice were treated with either AraC + G-CSF or carrier alone. There were progressive increases in the nucleated cell counts (NCCs) and Lin-c-kit^+^ cells in the spleen, PB, and liver by day 5 (Fig 1). Particularly in splenectomized mice, significant increases in the NCCs and Lin-c-kit^+^ cell counts were noted in the PB and liver; NCCs in the PB reached a maximum (approximately 8 x 10^6 cells/μL) on day 5. However, the increase in NCCs of the PB was mostly due to an increase in granulocytes (60% to 80% of the NCCs). The frequency of Lin-c-kit^-^ cells in the PB on day 5 was low (0.2% to 0.3%) despite splenectomy. After ceasing the administration of G-CSF, the NCCs in the spleen, PB, and liver decreased sharply from day 5 to 9. In the BM, Lin-c-kit^-^ cells decreased on day 1, increased on day 3 (to an almost normal level), and then decreased again. These Lin-c-kit^-^ cells maintained low levels until day 9 despite the increasing NCCs. Control mice that were injected with 0.1% BSA (carrier of G-CSF) showed no change in the distribution of these
cells in the various tissues irrespective of splenectomy (data not shown).

Frequency of blood progenitor cells. The percentages of CFU-C and CFU-S on day 12 in the extramedullary sites increased 20- to 80-fold on day 5 (Table 1), although those in the BM decreased to approximately half. In the splenectomized mice, slightly more CFU-C and CFU-S per 10⁶ cells were detected in the PB and liver (data not shown).

MRA assays were performed to evaluate more immature hematopoietic cells, because it has been reported that MRA reflects activities of pre-CFU with low activities of CFU-C and CFU-S. As shown in Fig 2, on day 0 (before treatment), cells with MRA were found mainly in the BM but some were found in the spleen, whereas there were very few cells with MRA in the PB and liver. However, 5 days after treatment, a higher MRA was found in the spleen, PB, and liver than in the BM. The PBSCs on day 5 showed a lower activity in MRA than the BMCs on day 0 (normal BMCs), probably due to the marked increase in granulocytes in the BM.

Surface markers of immature hematopoietic cells in mobilized PBSCs and BMCs. To examine the differences between immature hematopoietic cells (Lin- Sca-1⁺ or Lin- c-kit⁺ cells) in mobilized PBSCs and normal BMCs, three-color flow cytometric analyses were performed. Phenotypic analyses of these immature PBSCs in nonsplenectomized and splenectomized mice showed similar characteristics. Among Lin-Sca-1⁺ cells, mobilized PBSCs expressed slightly lower levels of VLA-4, LFA-1, and c-kit antigens (Ag) than did normal BMCs, although the level of Pgp-1 was similar to that of normal BMCs (Fig 3A). Lin-Sca-1⁺ cells in mobilized PBSCs did not express CD4 Ag, although those in BMCs showed a low CD4 expression (Fig 3B).

In the same way, among Lin-c-kit⁺ cells, we compared mobilized PBSCs with normal BMCs to examine the differences in hierarchical composition (Fig 3C). Both these cells displayed a high level of H-2K Ags. The frequency of cells expressing Sca-1 or Thy-1.2 Ag in Lin-c-kit⁺ cells was higher in mobilized PBSCs (about 60%) than in BMCs (about 20%). It is thought that CD71 Ag (transferrin receptor antigen) is expressed mostly on erythroid progeny, and that it is expressed more in proliferating cells than in quiescent cells. Less than 10% of Lin-c-kit⁺ cells in mobilized PBSCs expressed CD71 Ag, whereas greater than 20% of these cells in BMCs expressed CD71 Ag. Mobilized PBSCs showed lower fluorescent intensity in Rh123 staining than did BMCs, ie, 37.6% in the mobilized PBSCs and 26.0% in the BMCs was 25.7% in the spleen and liver (data not shown). Thus, Lin-c-kit⁺ cells of mobilized PBSCs showed more immature phenotypes than did those of BMCs.

**Hematopoietic activity of Lin-c-kit⁺Sca-1⁺(Thy-1.2⁺) cells in mobilized PBSCs.** Because of the threefold higher frequency of Sca-1⁺ or Thy-1.2⁺ populations in Lin-c-kit⁺ cells of mobilized PBSCs, we examined whether these fractions have primitive hematopoietic activity. As shown in Table 2A, 20% to 50% of the Sca-1⁺ or Thy-1.2⁺ population formed CFU-C colonies in the presence of IL-3 and Epo, and about 60% of Sca-1⁺ cells formed colonies in the presence of IL-3, SCF, IL-6, and Epo. In contrast, 10% to 20% of Sca-1⁺ cells formed colonies in the pres-

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**Table 1. CFU-C and CFU-S in Various Tissues Before and After Mobilizing Protocol**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CFU-C (10⁶)</th>
<th>CFU-S Day 12 (10⁶)</th>
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<tbody>
<tr>
<td>BM</td>
<td>207.5 ± 14.5</td>
<td>28.0 ± 5.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.7 ± 0.7</td>
<td>11.0 ± 1.8</td>
</tr>
<tr>
<td>PB</td>
<td>1.3 ± 0.3</td>
<td>23.0 ± 4.0</td>
</tr>
<tr>
<td>Liver</td>
<td>22.5 ± 4.8</td>
<td>132.5 ± 25.0</td>
</tr>
</tbody>
</table>

Test cells pooled from eight mice were assayed before (day 0) and after (day 5) AraC + G-CSF treatment. Data are shown as the mean ± SE.
of IL-3 and Epo, but synergistic effects with SCF and IL-6 were not seen. The Sca-1⁻ fraction showed higher BFU-E counts than the Sca-1⁺ fraction (35.2% of Sca-1⁻ cells vs 4.1% of Sca-1⁺ cells).

In the Sca-1⁻ cells, the CFU-S counts were 5.0 on day 8 and 53.0 on day 12. On the other hand, in the Sca-1⁺ cells, the CFU-S counts were 18.5 on day 8 and 3.2 on day 12. Thus, the ratio of CFU-S counts on days 12 to 8 was higher in the Sca-1⁻ cells than in the Sca-1⁺ (Table 2A). These characteristics of the Sca-1⁻ or Sca-1⁺ fraction in mobilized PBCs were compatible with those of the fractions in BMCs (Table 2B), as described previously. ¹⁻ Thy 1.2⁺ cells formed more CFU-S counts on day 12 than on day 8, whereas Thy 1.2⁻ cells (not Sca-1⁻ cells) had the considerable numbers of CFU-S on days 12. Greater enrichment of CFU-S on day 12 was obtained in the Sca-1⁺ cell population (Table 2A). Thy 1.2⁺ cells of the Lin⁻ c-kit⁺ fraction in BMCs showed similar CFU-S activity to the cells in mobilized PBCs (Table 2B).

It has recently been reported that P-HSCs are c-kit⁻ or c-kit¹⁻. We therefore attempted to examine the expression of c-kit antigen [c-kit⁻ or c-kit¹⁻] on primitive hematopoietic cells in the Lin⁻ Sca-1⁺ fraction of the mobilized PBCs (Fig 3A). However, we failed to find any primitive cells with the c-kit⁻ or c-kit¹⁻ marker; histology results showed that c-kit⁻ or c-kit¹⁻ cells in the mobilized PBCs were committed hematopoietic cells (data not shown).

Morphologic aspects of Lin⁻ c-kit⁺ Sca-1⁺ cells of mobilized PBCs. Lin⁻ c-kit⁺ Sca-1⁺ cells of mobilized PBCs showed a lymphoid-like appearance in morphology on May-Grunwald-Giemsa staining (Fig 4A). A relatively scant cytoplasm, a large nucleus, and one to three smaller nucleoli were present in almost all of the cells. Electron microscopic studies showed that the greater part (80% to 90%) of this population was a relatively heterogeneous population and that the cells had a somewhat abundant cytoplasm and a round to kidney-shaped nucleus with slightly condensed chromatin only at the nuclear membrane and small marginal nucleoli (Fig 4B). The cytoplasm contained many mitochondria, several cisternae of endoplasmic reticulum (ER), free ribosome, Golgi apparatus, and a granule-like structure. These structural features suggest that most Lin⁻ c-kit⁺ Sca-1⁺ cells of mobilized PBCs are cells in the cycling phase (relatively high metabolic state), but not in the G₀ phase (the dormant state). However, the remaining cells (10% to 20%) of this population had a round nucleus with very little peripheral chromatin condensation and a thin rim of cytoplasm with poorly developed organelles (Fig 4C). Mitochondria were present but were not highly developed.

Allogeneic PBC transplantation. Long-term reconstituting ability (LTRA) was assessed using mobilized PBCs of C57BL/6J. After collecting the low-density PB from B6 mice on day 5 after AraC + G-CSF treatment, T cells were removed using anti-CD4 and CD8 MoAbs plus rabbit complement; this resulted in a reduction in T cells (CD4⁺ and CD8⁺ cells) from 18% to 6%. These mobilized PBCs (PBSCs; 7 × 10⁶) were transferred into lethally irradiated C3H mice. The PBSCs (7 × 10⁶) contained Lin⁻ c-kit⁺ cell counts equivalent to 1 to 2 × 10⁵ normal BMCs, which are capable of reconstituting allogeneic recipients.

The survival rate of [B6→C3H] chimeric mice after PBSC transplantation was 84% 24 weeks after transplantation; this rate was not significantly different from that of BMC transplantation (Fig 5). The WBC and platelet counts gradually increased 2 weeks after transplantation, and the WBC counts remained high (15,000 to 20,000/µL) from 8 to 24 weeks. Leukocytosis was also seen in the chimeric mice after BMC transplantation.

The survival rate of [B6→C3H] chimeric mice after PBSC transplantation was 84% 24 weeks after transplantation; this rate was not significantly different from that of BMC transplantation (Fig 6). No obvious symptoms of GVHD were detected.

H-2 typing showed that cells in the BM, spleen, PB, and lymph nodes (LN) in [B6→C3H] chimeric mice are almost entirely of donor H-2² origin (≥95%) from 2 to 24 weeks after transplantation. Myelomonocytic cells (Gr-1⁻ or Mac-1⁻), B cells (B220⁺), T cells (Thy 1.2⁻), and erythroid cells (CD71⁺) were almost all derived from the donors even 24 weeks after transplantation (Fig 7A).

On flow cytometric analyses using the BMCs of [B6→C3H] mice, Lin⁻ c-kit⁺ and Lin⁻ Sca-1⁺ cells were mostly donor-derived (approximately 99% and 96%, respectively) even 24 weeks after transplantation (Fig 7B). The BMCs in chimeric mice from 5 to 24 weeks after transplantation had almost the same CFU-C counts as normal BMCs (Fig 8). The CFU-S counts gradually increased from 5 to 24 weeks. Although the ratio of CFU-S on day 12 to day 8 was 0.61 at 5 weeks, this ratio increased to 1.04 at 12 weeks, and the CFU-S counts on day 12 had reached near normal levels by 24 weeks (Fig 8), indicating the strong self-renewal capacity of PBSCs in chimeric mice.

DISCUSSION

In this study, we examined the changes in the distribution of mobilized hematopoietic cells in mice after AraC plus G-
Fig 3. Comparison of surface markers between mobilized PBCs and normal BMCs. Lin-Sca-1 cells in the BMCs and the mobilized PBCs were stained with either (A) VLA-4, LFA-1, Pgp-1, and c-kit (ACK-4) or (B) CD4. (C) Lin-c-kit (ACK-4) cells in the BMCs and the mobilized PBCs were stained with H-2K, Sca-1, Thy1.2, CD71, and Rh123. Values represent the percentage of positive or negative population within Lin-c-kit cells. Lin in (A) and (C) represents B220-, Gr-1-, Mac-1-, CD4-, and CD8-.

Values from two separate experiments are shown.

CSF treatment; it is well known that AraC (a potent inhibitor of DNA synthesis extensively used in leukemic patients) elicits an active regenerative process after the initial marrow damage. AraC is a cytotoxic agent that injures the P-HSCs less than other cytotoxic drugs such as cyclophosphamide and busulfan. G-CSF has also been found to be an effective mobilizing factor. In the nonsplenectomized mice, there were remarkable increases in the numbers of cells with Lin-c-kit markers, cells with CFU-C and CFU-S activities, and cells with MRA in the spleen, PB, and liver on day 5, whereas these immature hematopoietic cells decreased in the BM on day 5. In the BM, a transient increase in Lin-c-kit cells was seen on day 3. This transient increase in Lin-c-kit cells in the BM was also shown in the SCF-treated mice. These results indicate the mobilization of HSCs and/or progenitor cells from the BM to the PB rather than de novo generation of these cells within circulation blood. Grzegorzekowski et al. directly showed the mobilization of CFU-GM and CFU-GEMM from the BM after in vivo injection of rhIL-7 into mice using Sr. This is a β-emitting, bone-seeking radionuclide substituting for calcium molecules in the bone and selectively induces BM aplasia. In the splenectomized mice, the number of Lin-c-kit cells in the PB or liver increased to a level 4 or 2 times higher, respectively, than in the nonsplenectomized mice. There are two possible roles of the spleen in the mobilization of PBSCs:
Table 2. CFU Assays of Lin– c-kit+ Cells

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>CFU-C (×10³)</th>
<th>CFU-S (×10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-3 + Epo</td>
<td>IL-3 + Epo + SCF</td>
</tr>
<tr>
<td>A. PBCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sca-1−</td>
<td>145.0 ± 10.1</td>
<td>163.8 ± 11.5</td>
</tr>
<tr>
<td>Sca-1+</td>
<td>252.5 ± 13.9</td>
<td>417.5 ± 21.5</td>
</tr>
<tr>
<td>Thy1.2−</td>
<td>123.0 ± 6.8</td>
<td>ND</td>
</tr>
<tr>
<td>Thy1.2+</td>
<td>270.0 ± 11.5</td>
<td>ND</td>
</tr>
<tr>
<td>B. BMCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sca-1−</td>
<td>226.0 ± 13.5</td>
<td>378.2 ± 30.0</td>
</tr>
<tr>
<td>Thy1.2+</td>
<td>225.5 ± 16.9</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Lin− cells were removed from mobilized PBCs (A) or BMCs (B) using immunomagnetic beads. Sca-1− or Sca-1+ cells in the c-kit− population were sorted. Thy1.2− or Thy1.2+ cells were prepared similarly. CFU-C assays were performed in the presence of various cytokines at the concentrations as described in Material and Methods, and CFU-C counts were scored on day 7. CFU-S assays were performed using lethally irradiated B6 mice. Data are shown as the mean ± SD.

Abbreviation: ND, not done.

Fig 4. Morphologic characteristics of Lin− c-kit (ACK-4)+Sca-1− cells of mobilized PBCs. (A) Small lymphoid-like cells were stained with May-Grünwald-Giemsa. One to three smaller nucleoli are present in almost all the cells. (B) Most (80% to 90%) of the cells had somewhat abundant cytoplasm and a round-shaped to kidney-shaped nucleus. The cytoplasm contains free ribosome, granule-like structures (Gr), rough ER cisternae (E), Golgi apparatus (Gli), and many mitochondria (M) (original magnification × 12,000). The insert is at a high magnification (original magnification × 36,000). (C) The remaining (10% to 20%) cells had a round nucleus with very little peripheral chromatin condensation and an obscure nucleolus and a thin rim of cytoplasm with poorly developed organelles (original magnification × 18,000).
Characterization of Mouse PBSCs

Fig 5. Hematopoietic recovery in PB after allogeneic PBSC transplantation (B6→C3H). C3H mice were lethally irradiated with 9.0 Gy and injected with 7 x 10^6 low-density, T-cell-depleted PBSCs (+). The WBC counts, Hct level, and platelet counts in the PB were measured from 2 to 24 weeks after transplantation, as described in the Materials and Methods. Data represent the mean ± SD of four or more mice. Data in the PB of normal mice (○) are shown.

Fig 6. Survival rate in [B6→C3H] chimeric mice reconstituted with normal BMCs (1.5 x 10^7 cells) or PBSCs (7 x 10^6 cells).

Fig 7. Flow cytometry analyses in the various tissues 24 weeks after allogeneic PBSC transplantation [B6→C3H]. (A) Cells in the various tissues were stained with FITC-H-2K^b (donor type) and PE-Gr-1^+ Mac-1, B220, Thy1.2, or CD71 MoAbs. BMCs of C3H mice were used as a negative control. (B) Hematopoietic progenitor cells (Lin^−c-kit^+ Lin^−Sca-1^+) were stained with FITC-H-2K^b (donor type). The percentages of positive populations within these progenitor cells are shown. Representative data are shown.
members of the $\beta_1$ and $\beta_2$ integrin families of cell adhesion receptors and were shown to be expressed on immature hematopoietic cells and to be involved in the adhesion of these cells to BM stroma, although there is controversy as to whether LFA-1 is an absolute requirement for the early stage of hematopoiesis. The c-kit Ag has been shown to play a major role in the adhesion of mast cells to fibroblasts and of immature hematopoietic cells to stromal cells. It is possible that the lower expression of these adhesion molecules (VLA-4, LFA-1, and c-kit) in the Lin-Sca-1+ fraction of mobilized PBCs reflects the mobilization of PHSCs and progenitor cells. This notion deserves serious consideration, because it was reported that intravenous injection with saturating amounts of anti-VLA-4 antibody into pri-

mates induced a modest increase in circulating WBCs and a significant mobilization of CFU-C and CD34+ cells. Furthermore, we have found that Lin−Sca-1+ cells in mobilized PBCs contain virtually no CD4+ cells, in contrast to those in normal BMCs. Previous studies showed that P-HSCs in the BM express a low level of CD4 Ag, but cells in the B-cell lineage or in the T-cell lineage lose CD4 Ag early after commitment. Although the function of CD4 Ag on PHSCs is not clearly defined, it is reported that CD4 plays a role in the interaction of hematopoietic cells with their micro-

environment via the Ia antigen, similar to mature T cells. Therefore, the absence of any CD4 Ag expression in the Lin Sca-1+ cells of mobilized PBCs may reflect the mobilization of immature hematopoietic cells, as with the lower expression of adhesion molecules on mobilized PBCs.

We have found that Lin−c-kit+ cells in mobilized PBCs contain more cells with Sca-1+, Thy1.2+, CD71+, and Rh123d" markers than those in the normal BMCs. Particularly, the number of Sca-1+ (Thy1.2+) cells in Lin−c-kit+ population of mobilized PBCs was three times that in the BMCs. The Sca-1+ cells in the mobilized PBCs formed more CFU-C colonies in the presence of IL-3, Epo, SCF, and IL-6, and also more CFU-S colonies on day 12 than Sca-1− cells. However, Thy1.2b cells in mobilized PBCs or BMCs did not form as many CFU-S on day 12 as had Sca-1+ cells. This could be explained by the fact that P-HSC activity in the BM of Thy1.2b-genotype mouse strain (B6, etc) was ob-

served in both Thy1.2− and Thy1.2+ populations.

In electron microscopy, most (80% to 90%) Lin−c-kit+Sca-1− cells in the mobilized PBCs had many organelles (mitochondria, rER, granule-like structure, etc). These morphologic characteristics of mobilized PBCs indicate a high metabolic state (cells in the cycling phase). These cells probably belong to committed progenitor cells. However, 10% to 20% of this population contained only a few organelles with a higher nuclear cytoplasmic ratio. The latter cells had a similar structure to the cells in WGA+ fraction in the BM after 5-fluorouracil (5-FU) treatment (both in vivo and in vitro), although the nucleus of these WGA+ cells consists of more heterochromatin and fewer mitochondria. These cells probably show lower fluorescent intensity in Rh123 staining, which selectively stains mitochondrial membranes. It has also been reported that the Rh123−Lin−c-kit+Sca-1− cells in the BM are highly enriched for long-term repopulating cells (1 in 5 cells) and that 88% of the recipients of 10
such cells show greater than 1% donor cells in the peripheral blood at 26 weeks after transplantation.\textsuperscript{77} We are in the process of characterizing the function of Rh123\textsuperscript{Lin} c-kit\textsuperscript{+}Sca-1\textsuperscript{+} cells in the mobilized PBSCs by a competitive repopulation assay using Ly-5.1 and Ly-5.2 congenic mice. Previous studies have shown that c-kit Ag is highly expressed on P-HSCs and other progenitor cells in adult BMCs and fetal liver.\textsuperscript{17,18} However, in vivo blocking experiments using an antagonistic anti-c-kit MoAb (ACK-2) showed that a small but significant number of day 13 CFU-S, which were large mixed-type colonies, remained in the BM.\textsuperscript{18} It has also been shown that 5-FU-resistant dormany hematopoietic cells are c-kit\textsuperscript{+}.\textsuperscript{15} The lower expression of c-kit Ag on primitive hematopoietic cells was also reported in humans.\textsuperscript{19} We have very recently shown that only four Lin\textsuperscript{H-2K\textsuperscript{bop}} CD71\textsuperscript{+} cells in the BMCs after 5-FU treatment produce donor-derived cells in primary recipients over an 8-month period after transplantation and that the cells yield repopulation with detectable levels of primary donor-derived cells in secondary recipients.\textsuperscript{20} These P-HSCs were found to be Sca-1\textsuperscript{+}, Thy1\textsuperscript{+}, but c-kit\textsuperscript{+} or c-kit\textsuperscript{-}. We have just found that the 5-FU-resistant P-HSCs in the G\textsubscript{0} phase are c-kit\textsuperscript{-}; however, the P-HSCs show c-kit\textsuperscript{+} when the cells enter the cell cycle (manuscript in preparation). Although the Lin\textsuperscript{-}Sca-1\textsuperscript{-} fraction of our mobilized PSCs contained c-kit\textsuperscript{+} and c-kit\textsuperscript{-} populations, most of the c-kit\textsuperscript{+} or c-kit\textsuperscript{-} cells were contaminated by committed hematopoietic cells (morphologically). Further investigation of the relationship between the c-kit expression of P-HSCs and cell cycles is required.

It has been shown using various markers, such as Ly-5 alleles,\textsuperscript{25,51} neo\textsuperscript{+} gene,\textsuperscript{5} MDR-1 gene,\textsuperscript{8} and Y chromosome,\textsuperscript{52} that PBSCs have LTRA in the transplantation between congenic animals. However, it should be clarified whether these PBSCs can reconstitute allogeneic mice across MHC barriers. As we have shown in the present study, PBSCs had LTRA (24 weeks) in [B6–C3H] chimeric mice, although longer (>1 years) observation is necessary. In addition, the chimeric mice showed immunologic functions similar to mice that received BMCs, even 24 weeks after transplantation (Yamamoto et al, manuscript in preparation). This suggests the validity of allogeneic PBSC transplantation.

Autografting with PBSCs has become almost routine in the management of selected patients with leukemia and some solid tumors due to two major advantages of autografting PBSCs: one is rapid neutrophil and platelet recovery, and the other is easy harvesting of PBSCs without the risk of general anesthesia. If mobilized PBSCs contain P-HSCs, which can reconstitute even allogeneic hosts and do not induce severe GVHD, allogeneic PBSC transplantation can be expected to become a valuable strategy for the treatment of various diseases.\textsuperscript{9–10}

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