In Vivo and In Vitro Stem Cell Function of c-kit– and Sca-1–Positive Murine Hematopoietic Cells

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c-kit is expressed on hematopoietic stem cells and progenitor cells, but not on lymphohematopoietic differentiated cells. Lineage marker-negative, c-kit-positive (Lin–c-kit+) bone marrow cells were fractionated by means of Ly6A/E or Sca-1 expression. Lin–c-kit+ Sca-1+ cells, which consisted of 0.08% of bone marrow nucleated cells, did not contain day-8 colony-forming units-spleen (CFU-S), but 80% were day-12 spleen colonies, but they could not rescue the lethally irradiated mice and reconstituted hematopoiesis. On the other hand, 2 × 10^5 of Lin–c-kit+Sca-1- cells formed 20 day-8 and 11 day-12 spleen colonies, but they could not rescue the lethally irradiated mice. These data indicate that Lin–c-kit+Sca-1- cells are primitive hematopoietic stem cells and that Sca-1+ cells do not contain stem cells that reconstitute hematopoiesis. Lin–c-kit+Sca-1- cells formed no colonies in the presence of stem cell factor (SCF) or interleukin-6 (IL-6), and only 10% of them formed colonies in the presence of IL-3. However, approximately 50% of them formed large colonies in the presence of IL-3, IL-6, and SCF. Moreover, when single cells were deposited into culture medium by fluorescence-activated cell sorter clone sorting system, 40% of them proliferated on a stromal cell line (PA-6) and proliferated for more than 2 weeks. In contrast, 15% of the Lin–c-kit+Sca-1- cells formed colonies in the presence of IL-3, but no synergistic effects were observed in combination with SCF plus IL-6 and/or IL-3. Approximately 10% proliferated on PA-6, but most of them degenerated within 2 weeks. The population ratio of c-kit+Sca-1- to c-kit+Sca-1- increased 2 and 4 days after exposure to 5-fluorouracil (5-FU). These results are consistent with the relative enrichment of highly proliferative colony-forming cells by 5-FU. These data show that, although c-kit is found both on the primitive hematopoietic stem cells and progenitors, Sca-1+ cells are more primitive and respond better than Sca-1- cells to a combination of hematopoietic factors, including SCF and stromal cells.

**MATERIALS AND METHODS**

**Mice and bone marrow (BM) cell preparation.** The revised terminology for the Ly-5 alleles of the leukocyte common antigen (CD45), as provided by Morse et al., has been adopted in this report. Eight- to 15-week-old C57BL/6 (Thy-1.2, Ly-5.2) mice were obtained from Clea Japan Inc (Tokyo, Japan). C57BL/6-Ly-5.1-Pep3b (Thy-1.2, Ly-5.1) breeding pairs were provided by Dr Toshitada Takahashi (Aichi Cancer Center, Research Institute, Nagoya, Japan) and thereafter were bred and maintained in laminar flow housings at our animal facility. For some experiments, we administered 5-fluorouracil (5-FU; Adria Laboratories, Colombus, OH) through the tail vein at a dosage of 150 mg/kg body weight some days before the experiment. The mice were killed at 1, 2, or 7 days after the injection.

**HGFs.** Rat recombinant purified SF with a specific activity of 1.56 mg/mL was provided by Dr Kristina M. Zsebo (Amgen Biologics, Thousand Oaks, CA). Recombinant murine interleukin-3 (IL-3) was provided by Dr Ken-Ichi Arai (DNAX, Palo Alto, CA). Human purified IL-6 was provided by Ajinomoto Inc (Kawasaki, Japan). Human recombinant erythropoietin (Epo) was provided by Dr Masatsugu Ueda (Snow-Brand Milk Product Co, Ishibashi, Tochigi-ken, Japan). Human recombinant granulocyte CSF (GM-CSF; specific activity, 3.8 × 10^8 U/mg protein), and purified human recombinant macrophage-CSF (M-CSF; specific activity, 1 to 2 × 10^8 U/mg protein) were provided by Chugai Pharmaceutical Co (Kawasaki, Japan), Sumitomo Pharmaceutical Co (Osaka, Japan), and Moringa Milk Industries (Zama, Kanagawa-ken, Japan), respectively. Purified human recombinant IL-1α (specific activity, 4 to 6 × 10^6 U/mg protein) was provided by Dainippon Pharmaceutical Co (Osaka, Japan). The standard
concentrations of the HGFs used in our culture were as follows: rat SCF 100 ng/mL; murine IL-3 200 U/mL; human IL-6 20 ng/mL; human Epo 2 U/mL; human GM-CSF 20 ng/mL; murine M-CSF 100 U/mL; human IL-3 30 U/mL.

**Antibodies.** Anti-B220 (RA3-6B2), Mac-1 (M1/70), Gr-1 (RB6-8C5), L3T4 (GK1.5), Ly-2 (53-6.72), and TER119 (erythroid lineage marker) were used as lineage markers. Antibody specific for the Ly-6A/E molecules (clone E13 161-7, termed stem cell antigen or Sca-1) were kindly provided by Dr. Y. Aihara (Department of Pediatrics, Yokohama City University, Yokohama, Japan). A2O.1 (anti-Ly-5.1) and 104.2 (anti-Ly-5.2) were provided by Dr. Shoji Kimura (Sloan-Kettering Memorial Cancer Center, New York, NY) and Dr. Hidetaka Yakura (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan), respectively. Antibodies were coupled to fluorescein isothiocyanate (FITC) or biotin by the standard method. A hybridoma producing anti-c-kit-encoded molecule monoclonal antibody (MoAb) (ACK-2) was established from a rat immunized with IL-3-dependent normal mast cells. The purified MoAb (ACK-2) was conjugated with allophycocyanin (APC) for cell sorting. FITC-conjugated Thy-1.2 and phycocerythrin-coupled streptavidin were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA).

**Cell staining and analysis.** BM cells were flushed from femurs with phosphate-buffered saline (PBS) containing 3% fetal calf serum (FCS; Flow Laboratories, North Ryde, New South Wales, Australia). The suspension was washed and passed through a 100 mesh-per-inch stainless steel grid to produce a suspension of single cells. Red blood cells were lysed with an ammonium chloride-potassium buffer. Cells were incubated with ACK-2-APC, Sca-1-FITC, and biotinylated lineage markers (Lin; B220, Mac-1, Gr-1, L3T4, Ly-2, and TER119). After 20 minutes of incubation on ice, the BM cells were washed twice and incubated with phycoerythrin-streptavidin for 20 minutes on ice. The cells were washed twice and resuspended in PBS supplemented with 5% FCS, 0.02% NaN₃, and 1 μg propidium iodide (PI)/mL. The negative controls were unstained cells or cells stained with only second antibodies. Stained cells were analyzed and sorted on a FACStarplus (Becton Dickinson). Multiparameter data were collected and analyzed using FACS-DESK (Version 1.2) run on a digital micro VAX-II GPX, configured as previously described. The fluorescence intensity of individual cells was measured as relative fluorescence units.

**In vitro colony assay.** Methylcellulose culture was performed using a modification of the technique described Iscove et al. In routine experiments, 1 mL of culture medium contained an appropriate number of fresh or sorted BM cells, 1.2% methylcellulose (Fisher Scientific, Norcross, GA), α-medium (Flow Laboratories), 30% FCS, 1% deionized bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO), 0.1 mmol/L 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, NY), and HGFs. The mixture was incubated in 35 mm nontissue culture dishes (Falcon Labware, Oxnard, CA) under a humidified 5% CO₂ atmosphere at 37°C. The number of colonies were scored after 8 days of culture using an inverted microscope. Colony types were confirmed by lifting them from the semisolid medium on day 8 of culture, and Cytospin (Shandon Southern Instruments Inc, Sewickley, PA) preparations were stained with May-Grünwald-Giemsa.

**Spleen colony assay.** The spleen colony assay of Till and McCulloch was applied. Unfractionated or sorted BM cells were injected into lethally irradiated mice (9.5 Gy total body irradiation) from a dual-¹³⁷Cs source at a dose of 1.0 Gy/min. The spleens were removed on days 8 or 12 after transplantation, fixed in Bouin’s solution, and macroscopically visible spleen colonies were counted. Secondary transfers of spleen cell suspensions were assayed 12 days after primary transplantation to determine pre-colony-forming units-spleen (pre–CFU-S). The number of day-12 spleen colonies in secondary animals was normalized to a donor spleen and to the number of cells (per 1 × 10⁷) injected into the primary animals.

**Long-term reconstitution of lethally irradiated mice.** Sorted BM cells from Ly-5.1 mice were injected into lethally irradiated Ly-5.2 mice. Several weeks after the transplantation, peripheral blood was obtained from the retro-orbital sinus and dual-stained using MoAbs specific for lineage marker for B cells (anti-B220), myeloid cells (anti-Gr-1 and anti-Mac-1), or T cells (anti-Thy-1.2) with antibodies to congeneric markers specific for donor hematopoietic cells (anti-Ly-5.1) and analyzed by FACStarplus.

**Coculture of HSCs with stromal cells.** PA-6 is a stromal cell line established from newborn mouse calvaria by Kodama et al. that supports the myelopoiesis. PA-6 cell line was cultured to confluence in 96-well microtiterplates (Costar, Cambridge, MA). HSCs were clone-sorted and cultured on this stromal layer with 0.1 mL of medium containing 10% FCS.

**RESULTS**

**Purification of primitive HSCs.** Figure 1 shows a three-color analysis of BM cells by c-kit, Sca-1, and a mixture of lineage markers (B220, Mac-1, Gr-1, L3T4, and TER119). The two-color analysis shows that the expression of c-kit and Sca-1 was quite reciprocal. However, when the lineage-marker-negative (Lin⁻) population (8.49% ± 1.89% of the BM) was gated, a c-kit and Sca-1 double-positive fraction was observed that was only 0.08% ± 0.05% of the BM. As we have previously proven that HSCs exist in the...
Lin^{-}c-kit^{+} fraction\textsuperscript{25} and Spangrude et al\textsuperscript{1} showed that primitive HSCs express the Sca-1 antigen, and the Lin^{-}c-kit^{+} fraction was further divided by the expression of Sca-1. Each population was sorted by FACStarplus and assayed for in vitro colony formation, CFU-S, and transplantation.

As shown in Table 1, 100 Lin^{-}c-kit^{+}Sca-1\textsuperscript{+} cells formed no colonies on day 8 and 7.9 ± 0.8 colonies on day 12. On the other hand, 2 × 10\textsuperscript{3} Lin^{-}c-kit^{+}Sca-1\textsuperscript{-} cells formed 20.0 ± 2.0 colonies and 11.0 ± 1.0 colonies on days 8 and 12, respectively.

The term "pre-CFU-S" implies that the cells associated with this activity are too primitive to form splenic colonies within a 12-day period but are capable of producing day-12 CFU-S as differentiating progenies.\textsuperscript{5,4} As shown in Table 2, only Lin^{-}c-kit^{+}Sca-1\textsuperscript{+} cells had high pre-CFU-S activity. The other fraction generated a few colonies in the second recipients, but flow cytometry using Ly-5 congenic mice showed that most colonies were derived from recipient cells (data not shown). At least 100 Lin^{-}c-kit^{+}Sca-1\textsuperscript{-} cells rescued the lethally irradiated mice (20 of 20 mice survived more than 60 days) and reconstituted hematopoiesis in the recipient mice 20 weeks after transplantation (Fig 2).

These data indicate that Lin^{-}c-kit^{+}Sca-1\textsuperscript{+} cells are highly purified primitive HSCs and that Lin^{-}c-kit^{+}Sca-1\textsuperscript{-} cells are more committed progenitors. Moreover, we confirmed that c-kit\textsuperscript{-} cells do not contain CFU-S and pre-CFU-S.

**Effects of SCF and other hematopoietic factors on primitive HSCs.** Sorted BM cells were cultured in the presence of SCF, IL-3, and/or IL-6 (Fig 3). Lin^{-}c-kit^{+}Sca-1\textsuperscript{+} cells formed no colonies in the presence of SCF alone and only 10\% of cells formed colonies in the presence of IL-3 alone. About 25\% of them formed colonies in the presence of IL-3 plus SCF, IL-6 plus SCF, or IL-3 plus IL-6, and 48\% of them formed colonies in combination with IL-3, IL-6, and SCF. Moreover, the size of the colonies increased significantly in the presence of SCF. Although no colonies were formed in the presence of SCF alone, there were apparent synergistic effects with IL-3 and IL-6.

On the other hand, 15\% of Lin^{-}c-kit^{+}Sca-1\textsuperscript{-} cells formed colonies in the presence of IL-3, but the number and the size of colonies did not significantly increase in the presence of IL-6 and/or SCF.

Sorted BM cells were cultured in the presence of other hematopoietic factors (Table 3). No colonies formed from the Lin^{-}c-kit^{+}Sca-1\textsuperscript{+} fraction in the presence of IL-1α, and a few colonies were formed in the presence of G-CSF, GM-CSF, or M-CSF alone, or IL-3 plus Epo. About half of them formed colonies in the presence of SCF, IL-3, and IL-6, and the number of colonies did not increase significantly when IL-1α, M-CSF, or IL-1α plus G-CSF plus GM-CSF plus M-CSF were added.

**Colony formation by clone-sorted HSCs in the presence of stromal cells.** Single clone-sorted Lin^{-}c-kit^{+}Sca-1\textsuperscript{+} or Lin^{-}c-kit^{+}Sca-1\textsuperscript{-} cells were cultured in the presence of a stromal cell line (PA-6). After a confluent PA-6 stromal layer was established on 96-well microtiter plates, HSC fractions were clone-sorted and single cells were cultured. As shown in Table 4, 40\% of Lin^{-}c-kit^{+}Sca-1\textsuperscript{-} cells formed colonies and they were maintained for more than 2 weeks until the stromal cells degenerated. On the other hand, 10\% of Lin^{-}c-kit^{+}Sca-1\textsuperscript{-} cells formed colonies within 1 week of culture, but most of them degenerated within 2 weeks.

**The effect of 5-FU on stem cell populations.** The primitive population of HSCs selectively survives by treating mice with a high dose of 5-FU, because the proliferating precursors are killed.\textsuperscript{36} BM cells of the 5-FU-treated mice were stained with c-kit, Sca-1, and the mixture of lineage markers and analyzed by FACStarplus (Fig 4). The c-kit\textsuperscript{-}Sca-1\textsuperscript{-} fraction was decreased more drastically than c-kit\textsuperscript{-}Sca-1\textsuperscript{-} fraction by 5-FU and recovered gradually 4 days after. The population ratio of Lin^{-}c-kit^{+}Sca-1\textsuperscript{-} cells to Lin^{-}c-kit^{+}Sca-1\textsuperscript{-} cells after 5-FU treatment were 0.03 (day 0), 0.15 (day 1), 1.20 (day 2), 1.03 (day 4), and 0.34 (day 7). The ratio increased and reached a plateau (more than 1.0) on 2 and 4 days after 5-FU administration. Lin^{-}c-kit^{+}Sca-1\textsuperscript{-} cells from days 2 and 4 post-5-FU formed colonies on PA-6 (Table 4) and the numbers of colonies were the same as those that were untreated.

**DISCUSSION**

In a previous study, we showed that c-kit is expressed in primitive HSCs and progenitors and plays an essential role in the early stages of hematopoiesis.\textsuperscript{25} In this study, we confirmed that c-kit\textsuperscript{-} cells do not contain day-12 CFU-S and pre-CFU-S. We further divided the Lin^{-}c-kit^{+} fraction according to the expression of the Ly6A/E antigen, using the Sca-1 antibody.\textsuperscript{4} About 80\% of Lin^{-}c-kit^{+}Sca-1\textsuperscript{+} (here-
Fig 2. Multiple lymphohematopoietic repopulation by Lin-c-kit+Sca-1+ cells. One hundred Lin-c-kit+Sca-1+ cells from Ly-5.1 mice were injected intravenously into lethally irradiated Ly-5.2 mice. Twenty weeks after injection, donor-derived (Ly-5.1) cells were detected in the peripheral blood and phenotyped by two-color FACS analysis. Approximately 95% of the peripheral blood leukocytes in these mice were derived from donor cells.

after referred to as Sca-1+ cells were day-12 CFU-S, when the f value (seeding efficiency) is estimated to be 0.1. This fraction did not contain day-8 CFU-S. A small number (100 cells) of cells protected mice from lethal irradiation, and they generated long-term repopulation of both myeloid and lymphoid lineages. Moreover, by retransplantation, we showed that Sca-1+ cells had high pre-CFU-S activity.

By contrast, Lin-c-kit+Sca-1- (hereafter referred to as Sca-1- cells) gave rise to both day-8 and day-12 spleen colonies, but 2 × 10^5 cells did not protect mice from lethal irradiation. Thus, Sca-1+ cells were proven to be highly enriched primitive HSCs, whereas Sca-1- cells were progenitors, because they did not contain self-renewal HSCs (pre-CFU-S), although they included CFU-S.

We compared the response of Sca-1+ and Sca-1- cells with hematopoietic factors and found significant differences between them. For Sca-1+ cells, SCF by itself was not a sufficient growth stimulus, but it had considerable synergistic effects in combination with IL-3 and/or IL-6. Moreover, the number of colonies did not increase more even after the addition of other hematopoietic factors such as IL-1α, M-CSF, and G-CSF, which affect early hematopoietic progenitors. On the other hand, although IL-3 stimulated the Sca-1- cells, IL-6 and SCF had no synergistic effect. These data indicate that the more primitive stem cells require combinations of factors such as SCF, IL-3, and IL-6, and that SCF preferentially affects the primitive stem cells. However, it remains to be clarified whether SCF is required for the proliferation of the primitive HSCs (Lin-c-kit+Sca-1+).

Table 3. Colony Formation by Sorted BM Cells in the Presence of Hematopoietic Factors

<table>
<thead>
<tr>
<th>Factors</th>
<th>No. of Colonies per 100 Cells</th>
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<tbody>
<tr>
<td>SCF</td>
<td>Lin-c-kit+Sca-1+</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>G-CSF</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>M-CSF</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>IL-3 + Epo</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>SCF + IL-3 + IL-6</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>SCF + IL-3 + IL-6 + IL-1α</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>SCF + IL-6 + IL-1α + M-CSF</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>SCF + IL-1α + IL-3 + IL-6 + G-CSF + GM-CSF + M-CSF</td>
<td>44 ± 2</td>
</tr>
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Mean ± standard deviation of representative data of three repeated experiments.
Table 4. Colony Formation by Clone-Sorted Lin- c-kit+Sca-1+ BM Cells on Stromal Cell Line (PA-6)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>No. of Colonies/96 Clone-Sorted Cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Lin-c-kit+Sca-1+</td>
</tr>
<tr>
<td>Untreated</td>
<td>38 ± 9</td>
</tr>
<tr>
<td>5-FU-treated day-2</td>
<td>43 ± 9</td>
</tr>
<tr>
<td>5-FU-treated day-4</td>
<td>37 ± 2</td>
</tr>
</tbody>
</table>

Mean ± standard deviation of three repeated experiments. Abbreviation: ND, not done.

kit +Sca-1+). Ikuta et al26 have reported that the population of these cells increases in the fetal liver of the SI mice that lack SCF.

Recently, Pleomacher et al37 and Weilbaecher et al38 reported that only primitive HSCs grow on stromal layers (Dexter culture)39 and that this can serve as a quantitative measure of HSC activity. We cultured single HSCs on a stromal cell line (PA-6) that supports hematopoiesis. Sca-1+ cells proliferated on PA-6 at a higher incidence and proliferated longer than Sca-1- cells. In previous studies,40 PA-6 produced M-CSF, IL-6, SCF, and transforming growth factor β constitutively and IL-1β, IL-11, G-CSF, and leukemia inhibitory factor upon stimulation with IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-7, and GM-CSF were not produced by PA-6 (T. Sudo, personal communication, April 1992). Although it is not clear whether stromal function is replaced by the combination of factors, coculturing single stem cells with a stromal cell line are useful for the primitive stem cell assay.

Cycling cells, including committed progenitor cells, are eliminated by 5-FU, whereas pluripotent HSCs are spared.36 Treatment of mice with high doses of 5-FU therefore resulted in a relative enrichment of stem cells.41,42 Flow cytometry showed that Sca-1- cells disappeared and recovered 2 and 4 days after 5-FU treatment, respectively. The Sca-1+ cell fraction was also decreased by 5-FU, although a very low number of cells remained in this fraction. However, these cells had pre-CFU-S (data not shown) and colony-forming activity on the stromal cells. The population ratio of Sca-1+ to Sca-1- cells was increased from 0.03 to 1.2 on days 2 and 4 by 5-FU. This indicates that relative enrichment of primitive HSCs was obtained 2 or 4 days after 5-FU administration, which is consistent with previous studies on colony formation by hematopoietic cells from 5-FU-treated mice.36,43

In conclusion, although c-kit is expressed from primitive HSCs to the committed progenitors, Sca-1+ cells are more primitive and respond to SCF and stromal cells better than Sca-1- cells.

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Fig 4. The effect of 5-FU on stem cell populations. BM cells of 5-FU–treated mice were stained with c-kit, Sca-1, and a mixture of lineage markers (Lin; B220, Mac-1, Gr-1, Ly-2, L3T4, and TER119), and analyzed by FACScan®. When the Lin- populations were gated, c-kit+Sca-1+ and c-kit+ Sca-1- fractions was observed. A c-kit+ Sca-1- fraction was decreased drastically by 5-FU and was recovered gradually 4 days after.
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In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells

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