Role of Flk-1 in mouse hematopoietic stem cells

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Abstract It was reported that human hematopoietic stem cells in bone marrow were restricted to the $CD34^+KDR^+$ cell fraction. We found that expression levels of Flk-1, a mouse homologue of KDR, were low or undetectable in mouse Lin^-c-$Kit^+Sca\text{-}1^+CD34^{low/-}$ cells as well as $Hoechst33342^-$ cells (side population), which have long-term reconstitution capacity. Furthermore, neither Flk-$1^+CD34^{low/-}$ cells nor Flk-1^+CD34^+ cells had long-term reconstitution capacity in mouse. Taken together with other observations using Flk-1-deficient mice, these results indicate that Flk-1 is essential for the development of hematopoietic stem cells in embryo but not for the function of hematopoietic stem cells in adult mouse bone marrow.

Key words: KDR; Flk-1; Stem cell; CD34

1. Introduction

Studies on hematopoietic stem cells have been hindered by lack of a definitive positive marker. The membrane phosphoglycoprotein CD34 has been used as a useful marker of human hematopoietic stem/progenitor cells, and various types of colony-forming activity in bone marrow, cord blood, and mobilized peripheral blood have been proven to be included in the $CD34^+$ population in human [1,2]. Clinical transplantation studies using enriched $CD34^+$ bone marrow cells have also demonstrated the presence of hematopoietic stem cells with long-term reconstitution ability within this fraction [3,4]. Based on these findings, the current experimental and clinical strategies for the enrichment of human hematopoietic stem cells rely on the positive selection of the CD34 antigen. However, studies on the mouse system showed that hematopoietic stem cells with long-term reconstitution capacity were present in the $CD34^{low/-}$ cell population rather than in $CD34^+$ cells [5,6]. Actually, a single mouse Lin^-c-$Kit^+Sca\text{-}1^+CD34^{low/-}$ cell transplanted into lethally irradiated mice was able to sustain long-term multilineage engraftment [5]. Ito et al. [7], however, recently reported that developmental changes of CD34 expression from the positive to negative state took place between 7 and 10 weeks of age in mouse long-term engrafting cells, and that about 20% of adult hematopoietic stem cells remained $CD34^+$.

Recently, Ziegler et al. [8] reported that vascular endothelial growth factor receptor KDR/VEGFR2, Flk-1 in mice, is a major functional marker for postnatal hematopoietic stem cells in human. They described that human pluripotent hematopoietic stem cells in bone marrow were restricted to the $CD34^+KDR^+$ cell fraction, and that lineage-committed hematopoietic progenitor cells were in the $CD34^+KDR^-$ subset. The KDR was predominantly expressed in endothelial cells [9], and the mRNA was detected in human hematopoietic stem cells and megakaryocytes [10]. On the other hand, it has been suggested that Flk-1 plays an important role in embryonic hematoangiogenesis in mouse [11,14]. Flk-1 was expressed in the blood islands in the yolk sac of embryos [12] as well as in endothelial cells of the vascular sprouts and branching vessels of embryonic and early postnatal brain [11]. Targeted gene disruption studies demonstrated that Flk-1 was required for initiation of hematolymphopoiesis and vasculogenesis [13], implying that Flk-1 is required for generation of hemangioblasts, the stem cells for both hematolymphopoietic and endothelial lineage [14]. It is not known, however, whether Flk-1 is also expressed in hematopoietic stem cells in adult mouse bone marrow as shown in the human system.

Hematopoietic stem cells can also be identified based on their ability to efflux fluorescent dyes, such as Hoechst33342 and Rhodamine123. Goodell et al. [15] used Hoechst33342 staining of bone marrow cells to identify a minor fraction of side population (SP) cells highly enriched for repopulating activity. This SP phenotype identifies a primitive subset of stem cells in multiple mammalian species. Nowadays, both Rhodamine123 and Hoechst33342 are often used to identify the more limited hematopoietic stem cell population in SP cells [16].

Here we examined whether mouse hematopoietic stem cells in bone marrow, Lin^-c-$Kit^+Sca\text{-}1^+CD34^{low/-}$ cells and SP cells, express Flk-1, and whether Flk-1^+ cells have long-term reconstitution ability. We found that mouse hematopoietic stem cells are Flk-1^- or Flk-1^{low} and that Flk-1^+ cells had no long-term reconstitution capacity in mouse. We therefore concluded that Flk-1 is not essential for mouse hematopoietic stem cells. We discuss here the role of Flk-1 in hematopoietic stem cells.

2. Materials and methods

2.1. Mice

C57BL/6 mice (Ly-5.2) were purchased from Nihon SLC (Hamamatsu, Japan) and their congenic strain C57BL/6-Ly-5.1 Pep3b (Ly-5.1) was obtained from Sankyo Laboratory Service (Tsukuba, Japan). 8 to 10-week-old female mice were used as recipients in transplantation experiments.

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Abbreviations: SP, side population; PI, propidium iodide; PBS, phosphate-buffered saline; FCS, fetal calf serum

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2.2. Antibodies

Monoclonal antibodies against the following surface molecules were used: fluorescein isothiocyanate (FITC)-labeled anti-CD34 (RAM34), biotin-labeled anti-CD34 (RAM34), phycoerythrin (PE)-labeled anti-Flk-1 (Avas 12α1), anti-Sca-1 (E13-161.7), PE-labeled anti-Sca-1, allophycocyanin (APC)-labeled anti-c-Kit (2B8), FITC-labeled anti-Ly-5.1 (A20) antibodies (BD Pharmingen, San Diego, CA, USA). For isotype control, FITC-labeled rat IgG2a/κ and PE-labeled rat IgG2a/κ (BD Pharmingen #553929 and #553930) were used. Texas Red-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used to visualize unlabeled anti-Sca-1 antibody.

2.3. Preparation of bone marrow cells and depletion of lineage-positive cells

Bone marrow cells were prepared by flushing the femurs and tibias with phosphate-buffered saline (PBS) containing 2% inactivated fetal calf serum (FCS). The cell suspension was overlaid on Lymphoprep (Nycomed, Oslo, Norway) and centrifuged at $550\times g$ for 15 min. The low-density cells were harvested and washed with PBS. In some cases, bone marrow cells were depleted of lineage-positive cells using a magnetic particle concentrator (Dynal MPC-1; Dynal, Oslo, Norway). Cells were reacted with biotinylated anti-lineage antibodies (Mac-1, Gr-1, B220, CD4, CD8 and Ter119) (BD Pharmingen) for 30 min, absorbed to streptavidin magnetic beads (Dynabeads M-280; Dynal) for 30 min and separated on MPC-1 equipment for 2 min. The non-adherent (lineage-negative) cells were collected and stained with the subsequent antibodies as indicated.

2.4. Staining of Sca-1, c-Kit, CD34 and Flk-1

For five-color analysis, the nucleated cells (5×10^8 cells/ml) were suspended in staining medium (PBS containing 2% FCS and 0.05% NaN_3), and incubated with anti-Sca-1 antibody on ice for 30 min. After washing, the cells were incubated with Texas Red-conjugated donkey anti-rat IgG antibody on ice for 30 min. After washing, the lineage-positive cells were depleted as described above, followed by staining with PE-labeled anti-Flk-1, FITC-labeled anti-CD34 and APC-labeled anti-c-Kit antibodies on ice for 30 min and washed. Immediately prior to fluorescence-activated cell scanning (FACS) analysis, 1 μg/ml propidium iodide (PI) (Sigma, St. Louis, MO, USA) was added to the cell suspensions.

2.5. Preparation of SP cells

Bone marrow cells were suspended in PBS containing 2% FCS at the concentration of 10^6 cells/ml, incubated with 5 μg/ml Hoechst33342 (Sigma) at 37°C for 90 min and washed. When preparing Rhodamine123$^-$ SP cells, the Hoechst33342-stained cells were further incubated with 0.1 μg/ml Rhodamine123 (Sigma) at 37°C for 60 min. For staining of the cells with cell surface antigens, the cells suspended at 5×10^8 cells/ml in staining media were incubated with the appropriate antibodies on ice for 30 min. The cells were kept on ice until being analyzed. Immediately prior to FACS analysis or sorting, 1 μg/ml PI was added to the cell suspensions.

2.6. Flow cytometry

Cells were analyzed and sorted on a FACSVantageSE™ (Beckton Dickinson Bioscience, San Jose, CA, USA). Hoechst33342 was excited at 351 nm and its fluorescence emission was detected at two wavelengths using 424/44 (blue) and 675/20 (red) bandpass filters (Omega Optical, Brattleboro, VT, USA) [15]. A 560 nm short pass dichroic mirror (Omega Optical) was used to separate these emission wavelengths. Fluorescence from the Hoechst dye was acquired in linear scales. Rhodamine123 was excited at 488 nm and its fluorescence emission was detected with 530/30 bandpass filter (Omega Optical). Fluorescence from the Rhodamine dye was acquired in log scales. Fluorescence emission of FITC, PE, APC, Texas Red and PI was detected using standard filters.

2.7. Long-term reconstitution assays

The Ly-5.2 recipients were irradiated with 850 rad given in a single dose from an X-ray source operated at 150 kV delivering 90 rad/min. Progenitor cells sorted from Ly-5.1 donors were injected into the irradiated recipients along with 2×10^5 whole bone marrow cells from normal Ly-5.2 mice. All animal care was in accordance with institutional guidelines. For the analysis of reconstitution, mice were bled from the tail and assayed for the presence of Ly-5.1-positive cells. Blood of recipient mice was collected in PBS with 10 mM EDTA. Erythrocytes were eliminated by lysis with 155 mM NH_4Cl. The remaining cells were filtered through nylon mesh, stained with anti-Ly-5.1 antibody and analyzed by FACS.

3. Results and discussion

Ziegler et al. [8] recently described that human hematopoietic stem cells were restricted to the CD34$^+$KDR$^+$ cell fraction in bone marrow, and that lineage-committed hematopoietic progenitor cells were in the CD34$^+$KDR$^-$ subset. Therefore, we examined the expression level of Flk-1, a mouse homologue of KDR/VEGFR2, in mouse hematopoietic stem cells. First of all, the expression of Flk-1 was examined in Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^{low/-}$ cells. The Lin$^-$ bone marrow cells were gated on c-Kit$^+$Sca-1$^+$ cells (Fig. 1A), and Fig. 1B shows the dotplot of Flk-1 versus CD34 on these cells. The results indicated that Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^{low/-}$ cells were Flk-1$^-$ or Flk-1^{low}. These results suggest that Flk-1 is not a useful marker for isolation of mouse hematopoietic stem cells in bone marrow, and that Flk-1 is not essential for hematopoietic stem cells in mouse bone marrow.

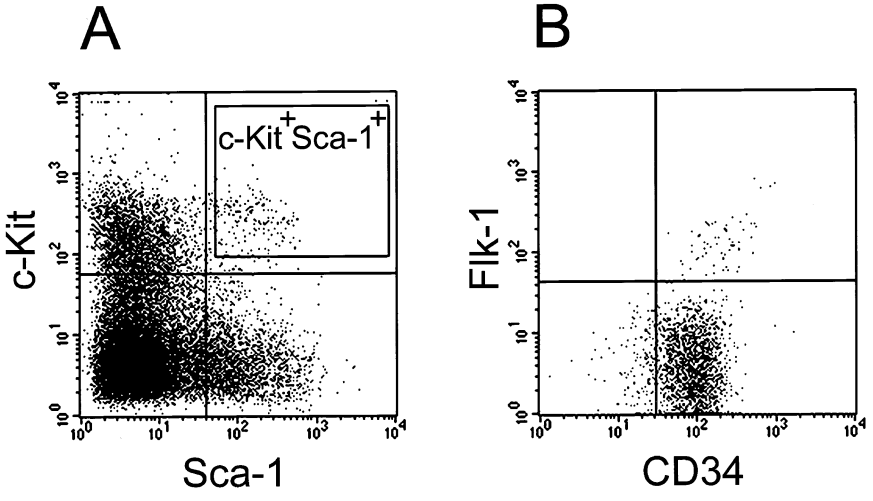

Fig. 1. Mouse Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^{low/-}$ cells are Flk-1$^-$ or Flk-1^{low}. A: Expression of Sca-1 and c-Kit on mouse Lin$^-$ bone marrow cells. The c-Kit$^+$Sca-1$^+$ cells were grated. Lin$^-$ bone marrow cells (2×10^5) were analyzed. B: Expression of Flk-1 and CD34 on c-Kit$^+$Sca-1$^+$ cells.

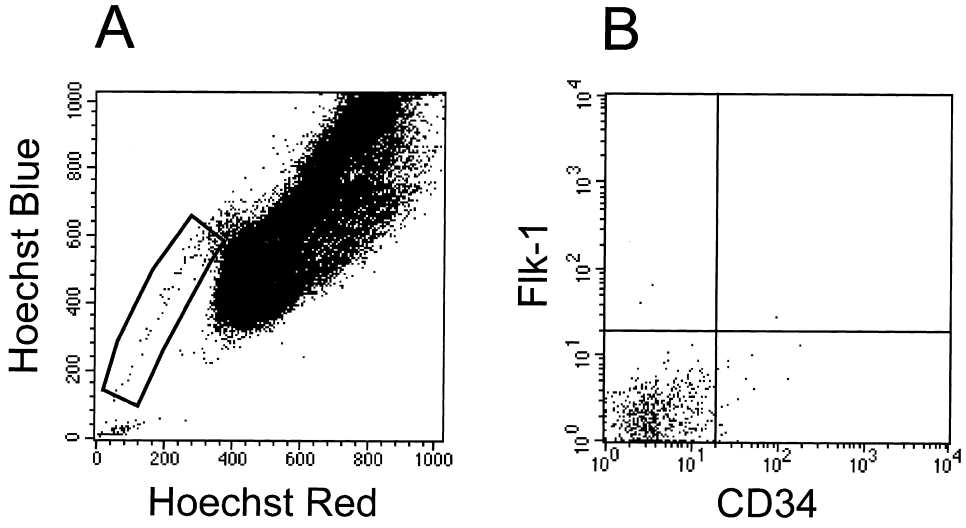

Fig. 2. Mouse SP cells are Flk-1$^{low/-}$. A: Mouse bone marrow cells stained with Hoechst33342 (4×10^6 cells). The SP cells were gated. B: Staining profile of Flk-1 versus CD34 on SP cells.

Similarly, the expression of Flk-1 and CD34 on mouse SP cells was examined. The bone marrow cells were gated on the SP region (Fig. 2A), and Fig. 2B shows the dotplot of Flk-1 versus CD34 of the SP cells. It was found that most of the SP cells (about 96%) were Flk-l$^{low/-}$CD34$^{low/-}$ and some (about 3%) were Flk-1$^{low/-}$CD34$^+$, and that SP cells did not contain Flk-1$^+$ cells at all. These results confirm that Flk-1 is not essential for mouse hematopoietic stem cells.

To test whether the Flk-1$^+$ population in mouse bone marrow cells has engraftment activity, Flk-1$^+$CD34$^+$ cells, Flk-1$^+$CD34$^{low/-}$ cells and Rhodamine$^{low/-}$ SP cells were prepared from Ly-5.1 mice. Fig. 3 shows the expression profile of Flk-1 and CD34 on mouse bone marrow cells. Only 0.27% of mouse bone marrow cells were Flk-1$^+$ cells, and Flk-1$^+$CD34$^+$ cells were constituted with only 0.09% of mouse bone marrow cells. The Flk-1$^+$CD34$^{low/-}$ cells were 0.18% of mouse bone marrow cells. The isotype control antibodies (FITC- and PE-labeled rat IgG2a/κ) did not stain these cells (data not shown). One hundred cells of each fraction were transplanted into lethally irradiated Ly-5.2 recipients. The purity of the transplanted cells was about 99% (data not shown). The level of engraftment was determined by measuring the percentage of donor peripheral blood nucleated cells 3 and 6 months after transplantation. While Rhodamine$^{low/-}$ SP cells, the more restricted population of SP cells, were successfully transplanted into the recipients over 6 months (18 out of 20 recipients) (Fig. 4A), neither Flk-1$^+$CD34$^+$ cells (Fig. 4B) nor Flk-l$^+$CD34$^{low/-}$ cells (Fig. 4C) were able to reconstitute lymphohematopoiesis at all (none of 20 recipients) at 3 months or 6 months after transplant. These results suggest that Flk-1$^+$ cells in mouse bone marrow have no long-term reconstitution ability and are not hematopoietic stem cells, and confirm that Flk-1 is not functionally essential for mouse hematopoietic stem cells.

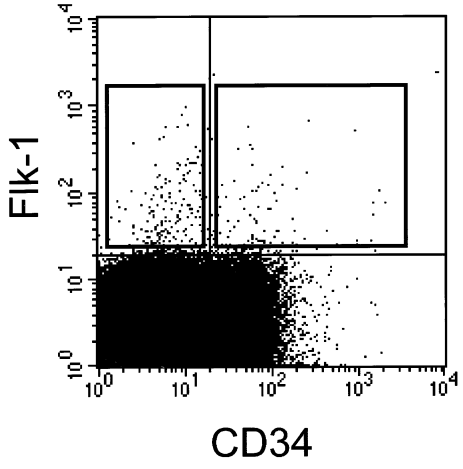

Fig. 3. Expression of Flk-1 and CD34 in mouse bone marrow cells. Bone marrow cells (2×10^5) were analyzed. The Flk-1$^+$CD34$^+$ cells and Flk-1$^+$CD34$^{low/-}$ cells used for transplant experiments are gated.

While it has been demonstrated that human hematopoietic stem cells were restricted to the CD34$^+$KDR$^+$ cell fraction [8], mouse hematopoietic stem cells were found not to express KDR homologue Flk-1. This finding is analogous to the case of CD34, which has been proved to be a crucial marker for isolation of human and mouse hematopoietic stem cells. These differences may be due to the fact that CD34 and KDR/Flk-1 are not common cell surface molecules that are biologically critical to be hematopoietic stem cells in any species. Therefore, identification of cell surface markers, which are definitively required for self-renewal of hematopoietic stem cells and/or for multipotency of hematopoietic stem cells, is awaited for the simple and easy isolation of hematopoietic stem cells in any species and for clinical applications.

Shalaby et al. [13] examined the developmental potential of Flk-1-deficient ES cells in chimeras, and found that these cells contribute neither to primitive nor definitive hematopoiesis. Further, cells lacking Flk-1 were unable to reach the correct location to form blood islands. They suggested that Flk-1 is involved in the movement of cells from the posterior primitive streak to the yolk sac and to the intraembryonic sites of early hematopoiesis. Hidaka et al. [17] also examined Flk-1-deficient ES cells for their hematopoietic potential in vitro during embryoid body formation or when cultured on the stromal cell line OP9. They found that Flk-1 plays a role in regulating

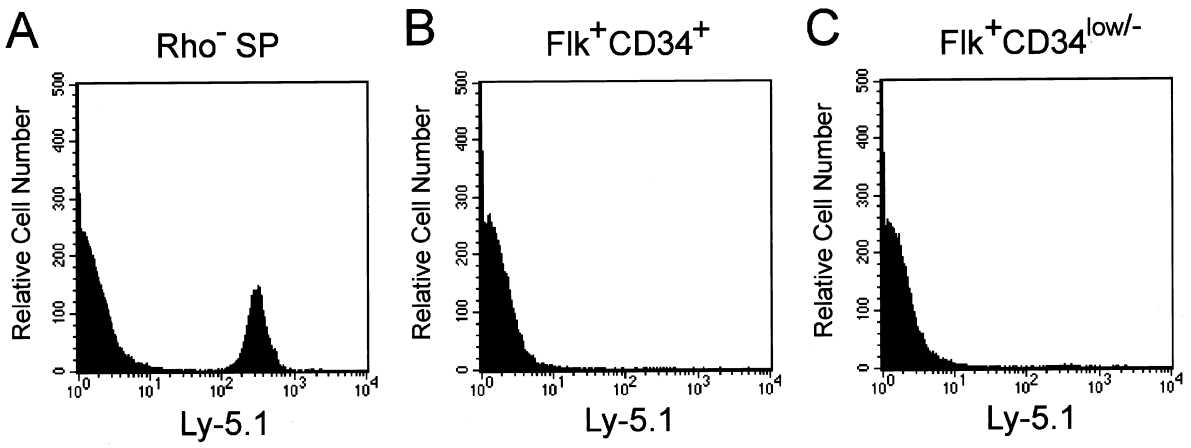

Fig. 4. No long-term reconstitution activity in Flk-1$^+$ bone marrow cells. The Rhodamine123$^{low/-}$ SP cells (A), Flk-1$^+$CD34$^+$ cells (B) or Flk-1$^+$CD34$^{low/-}$ cells (C) prepared from Ly-5.1 mice were transplanted into lethally irradiated Ly-5.2 recipients, and the representative data are shown. The level of engraftment was determined by measuring the donor-derived peripheral blood nucleated cells 6 months after transplantation.

the migration of early mesodermally derived precursors into a microenvironment, which is permissive for hematopoiesis. Kabrun et al. [12] investigated the expression pattern of the Flk-1 during mouse embryonic hematopoiesis, and found that embryos at day 8.5 of gestation contained Flk-1$^+$ hematopoietic precursors, whereas day 12.5 fetal liver contained few Flk-1$^+$ cells that showed little hematopoietic potential. Furthermore, they showed that all day 4 embryoid bodies-derived hematopoietic precursors, which were generated from ES cells differentiated in culture, were Flk-1$^+$, whereas greater than 95% of those found within the day 12 embryoid bodies were Flk-1$^-$. These results indicated that Flk-1 is expressed in early hematopoietic precursors that represent the onset of embryonic hematopoiesis. These findings show that Flk-1 may be essential for embryonic hematopoiesis but not for hematopoiesis in adult mouse bone marrow. Therefore, hematopoietic stem cells derived from adult mouse bone marrow do not necessarily have to express Flk-1 for their hematopoietic function, although KDR expression remains in human adult hematopoietic stem cells.

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