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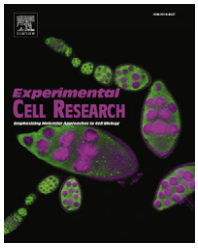

Research Article

CD45 ^{low}c-Kit ^{high} cells have hematopoietic properties in the mouse aorta-gonad-mesonephros region

Ikuo Nobuhisa[a,b,*], *Shoutarou Yamasaki*[b], *Ahmed Ramadan*[a,b], *Tetsuya Taga*[a,b,*]

[a]*Department of Stem Cell Regulation, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan*

[b]*Department of Cell Fate Modulation, Institute of Molecular Embryology and Genetics/Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, 860-0811, Japan*

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ABSTRACT

Long-term reconstituting hematopoietic stem cells first arise from the aorta of the aorta-gonad-mesonephros (AGM) region in a mouse embryo. We have previously reported that in cultures of the dispersed AGM region, CD45^{low}c-Kit^{+} cells possess the ability to reconstitute multilineage hematopoietic cells, but investigations are needed to show that this is not a cultured artifact and to clarify when and how this population is present. Based on the expression profile of CD45 and c-Kit in freshly dissociated AGM cells from embryonic day 9.5 (E9.5) to E12.5 and aorta cells in the AGM from E13.5 to E15.5, we defined six cell populations (CD45^{-}c-Kit^{-}, CD45^{-}c-Kit^{low}, CD45^{-}c-Kit^{high}, CD45^{low}c-Kit^{high}, CD45^{high}c-Kit^{high}, and CD45^{high}c-Kit$^{very\ low}$). Among these six populations, CD45^{low}c-Kit^{high} cells were most able to form hematopoietic cell colonies, but their ability decreased after E11.5 and was undetectable at E13.5 and later. The CD45^{low}c-Kit^{high} cells showed multipotency *in vitro*. We demonstrated further enrichment of hematopoietic activity in the Hoechst dye-effluxing side population among the CD45^{low}c-Kit^{high} cells. Here, we determined that CD45^{low}c-Kit^{high} cells arise from the lateral plate mesoderm using embryonic stem cell-derived differentiation system. In conclusion, CD45^{low}c-Kit^{high} cells are the major hematopoietic cells of mouse AGM.

Introduction

In mouse ontogeny, fetal hematopoiesis is initiated from blood islands of the yolk sac that wraps around embryos [1]. Later, emergence of cells containing the long-term repopulating activity in irradiated adult mice is found in the aorta-gonad-mesonephros (AGM) region at midgestation [2,3]. Hematopoiesis rapidly decreases in the AGM region and the major site of hematopoiesis mainly shifts to the fetal liver at E12.5 [4]. Around the same time, hematopoiesis was reported to transiently rise again in the yolk sac [5] and placenta [6,7]. After that, lymphopoiesis is observed in spleen and thymus, and hematopoiesis finally arrives at the bone marrow. Thus, the sites of hematopoiesis change during mouse ontogeny [8,9]. In a recent report, by the transient marking of a hematopoietic transcriptional factor AML1-expressing cells in the yolk sac of a E7.5 embryo, the marked cells are observed in AGM regions, fetal liver, and adult bone marrow at the stage when these tissues show hematopoiesis [10]. The results suggest that emergence of the hematopoietic stem cells (HSCs) initiates, at least in part, at the yolk sac. On the other hand, mice lacked a heartbeat by deletion of sodium–calcium exchanger (Ncx)-1 possessed hematopoietic progenitors in the placenta, yolk sac, and caudal half of embryos [11]. The data raise the

* *Corresponding authors at:* Department of Stem Cell Regulation, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. Fax: +81 3 5803 5814.
E-mail addresses: nobuhisa.scr@mri.tmd.ac.jp (I. Nobuhisa), taga.scr@mri.tmd.ac.jp (T. Taga).

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possibility that hematopoiesis arises in each hematopoietic tissue in a mouse embryo.

The AGM region is a primary source of definitive hematopoietic cells in the midgestation mouse embryo [3,12]. It is known that the common progenitors of hematopoietic cells and endothelial cells exist in the AGM. The presence of the marker proteins in adult bone marrow HSCs, Ly6A/E (Sca-1) and c-Kit, is observed in the aortic endothelium and the neighboring hematopoietic cell clusters [13,14], and the cell population that has the long-term repopulating activity shows expression of endothelial markers [13,15,16]. The expression of CD45, a marker of hematopoietic cells, is used as an index of fractionation of HSCs/hematopoietic progenitor cells in the AGM at midgestation. In previous studies, populations with negative and low-level CD45-expression have long-term repopulating activity [17], whereas the CD45-expressing population has this activity [16,18]. Our previous studies [19,20] showed that non-adherent cells from the primary culture of the dissociated AGM regions, which appear to be a reproduction of hematopoiesis in AGM regions [21], can be separated into at least $CD45^{low}c\text{-}Kit^{+}$, $CD45^{low}c\text{-}Kit^{-}$, and $CD45^{high}c\text{-}Kit^{low/-}$ populations based on the expression profile of CD45 and c-Kit. Also, only the $CD45^{low}c\text{-}Kit^{+}$ population possessed *in vitro* hematopoietic activity and *in vivo* ability to reconstitute multi-lineage hematopoietic cells. However, it remained unclear whether an equivalent cell population exists *in vivo* and how it behaves in accordance with development.

Here we report the presence of $CD45^{low}c\text{-}Kit^{high}$ cells having high hematopoietic activity in the mouse AGM. Their hematopoietic activity decreased later than E11.5 and disappeared at E13.5 and later. Various assays revealed the capacity of these $CD45^{low}c\text{-}Kit^{high}$ cells to develop multilineage hematopoietic cells. Moreover, hematopoietic activity in the $CD45^{low}c\text{-}Kit^{high}$ cells was enriched in the Hoechst side population (SP). The origin of the $CD45^{low}c\text{-}Kit^{high}$ cells was suggested to be the lateral plate mesoderm based on the analysis of a coculture of embryonic stem cells with stromal cells.

Materials and methods

Fractionation of AGM cells by flow cytometry

The AGM regions from E9.5 to E12.5 and the aorta from E13.5 to E15.5 were excised from ICR mice. The AGM regions were incubated in 1 mg/ml Dispase II (Roche, Mannheim, Germany) for 20 min at 37 °C. After washing in Hank's balanced salt solution (HBSS) containing 10% (v/v) fetal calf serum (FCS) and 250 μg/ml DNase I (Roche), the cells were treated with Cell Dissociation Buffer (Invitrogen, Carlsbad, CA, USA) for 20 min at 37 °C. Dissociated cells were then washed with HBSS containing 10% (v/v) FCS and 250 μg/ml DNase I. The washed cells were filtered through a 30 μm filter (Cell Trics; Perteck, Münster, Germany) and subjected to immunostaining with phycoerythrin (PE)-conjugated anti-mouse CD45 (30-F11), and allophycocyanin (APC)-conjugated anti-mouse c-Kit (2B8) (eBioscience, San Diego, CA, USA). In some experiments, the cells were also stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD31 (MEC13.3; Becton Dickinson, Lincoln, NJ, USA), anti-mouse CD49d (R1-2; eBioscience), anti-mouse vascular endothelial-cadherin (VECD1; [22,23]), or anti-mouse CD34 (RAM34; eBioscience). Stained cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% (v/v) FCS and 1 μg/ml propidium iodide (PI) (Calbiochem, Darmstadt, Germany) and analyzed by FACSAria and FACSVantage (Becton Dickinson, Lincoln, NJ, USA). The results of flow cytometry were analyzed with FlowJo (Three Star Inc., Ashland, Vermont, USA). $CD45^{-}c\text{-}Kit^{-}$ (P1), $CD45^{-}c\text{-}Kit^{low}$ (P2), $CD45^{-}c\text{-}Kit^{high}$ (P3), $CD45^{low}c\text{-}Kit^{high}$ (P4), $CD45^{high}c\text{-}Kit^{high}$ (P5), and $CD45^{high}c\text{-}Kit^{very\ low}$ (P6) cells were sorted by FACSAria and FACSVantage. Sorted cells were transferred to slides in a Cytospin (Shandon, Sewickley, PA, USA) centrifuged and examined morphologically after May-Grünwald-Giemsa staining.

Coculture with OP9 stromal cells

Sorted cells (2.5×10^2) from each population were seeded on OP9 stromal cells in α-minimal essential medium (MEM) supplemented with 10% (v/v) FCS, 50 ng/ml interleukin (IL)-3, 50 ng/ml stem cell factor (SCF), and 50 ng/ml thrombopoietin (TPO). After 4 days, hematopoietic cell colonies and colonies containing the cobblestone areas were counted.

A sorted single cell of $CD45^{-}c\text{-}Kit^{high}$ (P3) and $CD45^{low}c\text{-}Kit^{high}$ (P4) from E10.5 and E11.5 AGM was cocultured with OP9 stromal cells using a 48-well plate in α-MEM supplemented with 10% (v/v) FCS, 50 ng/ml IL-3, 50 ng/ml SCF, and 50 ng/ml TPO. After 2 weeks, cells in each well were recovered by pipetting and then cocultured with OP9 cells using a 12-well plate. Four weeks after the initial culture, colonies containing flattened and round cells or cobblestone-like areas were counted.

Semisolid colony-forming assays

Sorted cells (2.5×10^2) from each population were embedded in 1 ml of Methocult™ (M3434; StemCell Technologies, Vancouver, BC, Canada). Cells were cultured in 35-mm dishes at 37 °C for 10 days. Individual colonies were scored by the morphology.

Capacity of $CD45^{low}c\text{-}Kit^{high}$ cells to differentiate into B cells

$CD45^{low}c\text{-}Kit^{high}$ (P4) cells (5.0×10^2) sorted from E10.5 AGM cells were cocultured with OP9 in α-MEM supplemented with 10% (v/v) FCS, 100 ng/ml IL-7, and 100 ng/ml fms-like tyrosine kinase 3 ligand (Flt3L). After 10 days, non-adherent cells were incubated with PE-conjugated rat anti-mouse CD19 (1D3; Becton Dickinson) and analyzed by FACSAria.

Capacity of $CD45^{low}c\text{-}Kit^{high}$ cells to differentiate into endothelial cells

$CD45^{low}c\text{-}Kit^{high}$ (P4) cells (1.5×10^2) and single P4 cells sorted by E11.5 AGM were cocultured with OP9 cells in α-MEM supplemented with 10% (v/v) FCS, 50 ng/ml IL-3, 50 ng/ml SCF using a six-well plate (150 cells/well) and 48-well plate (1 cell/well), respectively. After 3 and 5 days respectively of culture, 50 ng/ml vascular endothelial growth factor (VEGF) was added to the culture. On day 7 of the initial culture, cells were stained with anti-mouse CD31 and control IgG antibodies.

Side population of the E10.5 AGM

The E10.5 AGM cells digested with Dispase II and Cell Dissociation Buffer were suspended at 10^6 cells/ml in DMEM supplemented with 2% (v/v) FCS and preincubated at 37 °C for 10 min. The cells