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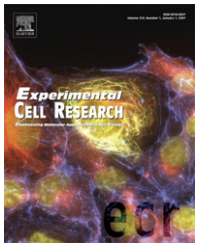

Research Article

Identification of a population of cells with hematopoietic stem cell properties in mouse aorta–gonad–mesonephros cultures

Ikuo Nobuhisa[a], *Naoki Ohtsu*[a], *Seiji Okada*[b], *Naomi Nakagata*[c], *Tetsuya Taga*[a,*]

[a]*Division of Cell Fate Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Kumamoto, Japan*
[b]*Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto, Japan*
[c]*Center for Animal Resources and Development, Kumamoto University, 2-2-1 Honjo, Kumamoto, Japan*

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ABSTRACT

The aorta–gonad–mesonephros (AGM) region is a primary source of definitive hematopoietic cells in the midgestation mouse embryo. In cultures of dispersed AGM regions, adherent cells containing endothelial cells are observed first, and then non-adherent hematopoietic cells are produced. Here we report on the characterization of hematopoietic cells that emerge in the AGM culture. Based on the expression profiles of CD45 and c-Kit, we defined three cell populations: $CD45^{low}$ c-Kit^{+} cells that had the ability to form hematopoietic cell colonies in methylcellulose media and in co-cultures with stromal cells; $CD45^{low}$ c-Kit^{-} cells that showed a granulocyte morphology; $CD45^{high}$ c-$Kit^{low/-}$ that exhibited a macrophage morphology. In co-cultures of OP9 stromal cells and freshly prepared AGM cultures, $CD45^{low}$ c-Kit^{+} cells from the AGM culture had the abilities to reproduce $CD45^{low}$ c-Kit^{+} cells and differentiate into $CD45^{low}$ c-Kit^{-} and $CD45^{high}$ c-$Kit^{low/-}$ cells, whereas $CD45^{low}$ c-Kit^{-} and $CD45^{high}$ c-$Kit^{low/-}$ did not produce $CD45^{low}$ c-Kit^{+} cells. Furthermore, $CD45^{low}$ c-Kit^{+} cells displayed a long-term repopulating activity in adult hematopoietic tissue when transplanted into the liver of irradiated newborn mice. These results indicate that $CD45^{low}$ c-Kit^{+} cells from the AGM culture have the potential to reconstitute multi-lineage hematopoietic cells.

Introduction

Definitive hematopoiesis, producing long-term repopulating hematopoietic stem cells (HSCs) in mouse embryos, initially arises from the aorta–gonad–mesonephros (AGM) region during the midgestation stage [1–3]. Within the AGM region, HSCs are mainly generated in the dorsal aorta [4,5]. Hematopoietic cells in clusters are found in the ventral wall of the dorsal aorta [6,7]. More recently, it has been shown that expression of Sca-1, which is one of the HSC markers, is observed in the endothelial cells along the dorsal aorta and overlaps with the endothelial markers [8]. It was shown that AGM cells positive for Runx-1 (also known as AML1, Cbfa2, and Pebp2aB) have hematopoietic activity [9]. It has also been shown that, during ES cell differentiation *in vitro*, hematopoietic cells are produced by co-culturing with stromal cells and ES-derived endothelial cells [10–13]. These results suggest that hematopoietic cells come from hemangioblast in mouse embryos in midgestation.

An *in vitro* primary culture system of the AGM region of midgestation mouse embryos is known to reproduce hematopoiesis [14]. Cells of the AGM region were cultured with cytokines such as stem cell factor (SCF), basic fibroblast growth factor (bFGF), and oncostatin M (OSM). After a few days in culture, adherent cells including endothelial cells were initially observed and then non-adherent hematopoietic cells were produced. Previous studies indicated the failure to produce

* *Corresponding author*. Fax: +81 96 373 6610.
E-mail address: taga@kaiju.medic.kumamoto-u.ac.jp (T. Taga).

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non-adherent cells in the AGM culture from mice with a genetic defect in either Runx-1 or c-Myb, each of which has an essential role in definitive hematopoiesis at the stage of HSC emergence [15,16].

We have previously reported the importance of the Ras/MAP kinase pathway in AGM hematopoiesis. The production of $CD45^+$ non-adherent cells from the AGM culture was blocked by the inhibition of the Ras/MAP kinase pathway by U0126, an Erk inhibitor. Moreover, introduction of Spred-2, which is a negative regulator of Ras/MAP kinase [17], reduced the emergence of $CD45^+$ non-adherent cells in the AGM culture [18]. Similar results were obtained by overexpression of Lnk, which suppresses the SCF-induced Erk phosphorylation through Gab-2 [19], in the AGM culture [20]. Spred-2- and Lnk-deficient mice showed enhanced AGM hematopoiesis. On the other hand, overexpression of a hematopoietic transcription factor GATA-2 in the AGM culture inhibited the appearance of $CD45^+$ non-adherent cells [21].

Previous studies on the expression of marker proteins could not distinguish between undifferentiated cells and differentiated cells in non-adherent cells from the AGM culture. In addition, long-term reconstitution activity of these undifferentiated cells from the AGM culture has not been elucidated. To clarify the fate of non-adherent cells in the AGM culture, we divided the cells into three populations based on their expression profile of CD45 and c-Kit; $CD45^{low}$ $c\text{-}Kit^+$, $CD45^{low}$ $c\text{-}Kit^-$, and $CD45^{high}$ $c\text{-}Kit^{low/-}$. We next investigated the functional properties of each population. $CD45^{low}$ $c\text{-}Kit^+$ cells were determined to be at an undifferentiated status and $CD45^{low}$ $c\text{-}Kit^-$ and $CD45^{high}$ $c\text{-}Kit^{low/-}$ cells were granulocytes and macrophages, respectively. When $CD45^{low}$ $c\text{-}Kit^+$ cells were co-cultured with OP9 stromal cells or freshly prepared AGM culture, all the three types in terms of the CD45 and c-Kit expression profile as described above emerged again. Moreover, $CD45^{low}$ $c\text{-}Kit^+$ cells had the potential to reconstitute *in vivo* hematopoiesis when injected into the liver of irradiated newborn mice. We concluded that HSCs were included in $CD45^{low}$ $c\text{-}Kit^+$ cells from the primary culture of AGM cells of midgestation mouse embryos.

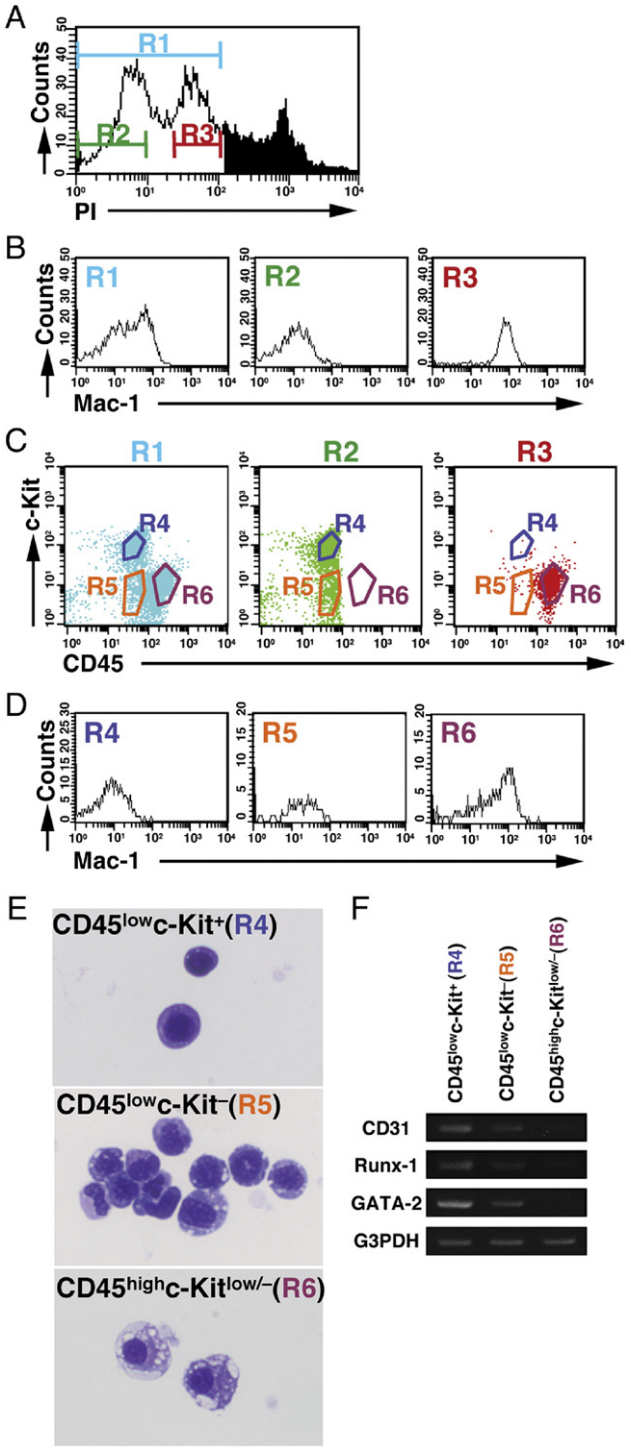

Fig. 1 – Characterization of the non-adherent cells from the AGM culture. (A) Non-adherent cells from the AGM culture were stained with PI and analyzed using flow cytometry (1×10^4 cells). The black area represents PI-positive cells. (B) The non-adherent cells were stained with PI, FITC–anti-Mac-1 antibody. The expression of Mac-1 was analyzed in R1, R2, and R3 populations, respectively. (C) The non-adherent cells were stained simultaneously with PI, FITC–anti-Mac-1 PE–anti-CD45, and APC–anti-c-Kit antibodies. R1, R2, and R3 populations were gated as indicated by the lines in panel A. The expression of CD45 and c-Kit were analyzed in each population. (D) R4, R5, and R6 populations were gated as indicated by the boxes in panel C. The expression of Mac-1 was analyzed in R4, R5, and R6 populations, respectively. (E) Morphologic analysis of the sorted cells from the AGM culture. R4, R5, and R6 populations sorted from the non-adherent cells of the AGM culture, according to the expression of CD45 and c-Kit. After sorting, cytospin preparation was stained with May-Grünwald/Giemsa. (F) Expression of CD31, Runx-1, and GATA-2 in sorted R4, R5, and R6 populations. Total RNA was extracted from sorted cells and then subjected to RT–PCR using specific primers for CD31, Runx-1, GATA-2, and G3PDH.

Materials and methods

AGM cultures

The AGM regions were excised at E11.5 from ICR mice. Trypsinized AGM cells were suspended in Dulbecco's