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Thrombopoietin contributes to the formation and the maintenance of hematopoietic progenitor-containing cell clusters in the aorta-gonad-mesonephros region

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ABSTRACT

In the midgestation mouse embryo, hematopoietic cell clusters containing hematopoietic stem/progenitor cells arise in the aorta-gonad-mesonephros (AGM) region. We have previously reported that forced expression of the Sox17 transcription factor in CD45^{low}c-Kit^{high} AGM cells, which are the hematopoietic cellular component of the cell clusters, and subsequent coculture with OP9 stromal cells in the presence of three cytokines, stem cell factor (SCF), interleukin-3 (IL-3), and thrombopoietin (TPO), led to the formation and the maintenance of cell clusters with cells at an undifferentiated state *in vitro*. In this study, we investigated the role of each cytokine in the formation of hematopoietic cell clusters. We cultured Sox17-transduced AGM cells with each of the 7 possible combinations of the three cytokines. The size and the number of Sox17-transduced cell clusters in the presence of TPO, either alone or in combination, were comparable to that observed with the complete set of the three cytokines. Expression of TPO receptor, c-Mpl was almost ubiquitously expressed and maintained in Sox17-transduced hematopoietic cell clusters. In addition, the expression level of c-Mpl was highest in the CD45^{low}c-Kit^{high} cells among the Sox17-transduced cell clusters. Moreover, c-Mpl protein was highly expressed in the intra-aortic hematopoietic cell clusters in comparison with endothelial cells of dorsal aorta. Finally, stimulation of the endothelial cells prepared from the AGM region by TPO induced the production of hematopoietic cells. These results suggest that TPO contributes to the formation and the maintenance of hematopoietic cell clusters in the AGM region.

1. Introduction

During ontogeny in the mouse, hematopoietic tissue is altered in yolk sac, the aorta-gonad-mesonephros (AGM) region, fetal liver, and bone marrow (BM) [1]. The AGM region, in which definitive hematopoiesis first arises at midgestation, contains intra-aortic hematopoietic clusters (IAHCs) along with endothelial cells of the dorsal aorta [2,3]. The IAHCs are derived from the hemogenic endothelium, which is a common progenitor for hematopoietic and endothelial cells. The cells in IAHCs express CD45, a marker of hematopoietic cells, CD31 and vascular endothelial-cadherin (VE-Cad), markers of endothelial cells, and c-Kit, a marker of hematopoietic stem cells (HSCs) [2,4–6]. The cells in IAHCs in the midgestational mouse embryo have the long-term repopulating ability in lethally irradiated mice, indicating that the cells of IAHCs include HSCs [4,6].

Sry-related high mobility group box 17 (Sox17) is a marker of the endoderm [7]. Recent studies have suggested that Sox17 is a key regulator of definitive hematopoiesis [3]. With the use of the culture system of embryonic stem (ES) cells and induced pluripotent stem (iPS) cells under hematopoietic differentiation-inducing conditions, the step-by-step analyses of hematopoietic differentiation have suggested a role of Sox17 in hematopoiesis: cells expressing Sox17 together with Flk-1 in embryoid bodies have a high ability to produce hematopoietic cells [8]. In the culture of Sox17-overexpressing human ES and iPS cells, hemogenic endothelium-like cells appear and are retained in the form of semi-adherent aggregates, and the elimination of Sox17 protein from the nuclei in Sox17-overexpressing hemogenic endothelium-like cells induces hematopoietic differentiation [9]. In the mouse embryo, long-term repopulating HSCs are enriched in cells expressing both Sox17 and VE-Cad in the E11.5 AGM region [8]. The expression

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level of Sox17 in the HSC population (lineage markers$^-$c-Kit$^+$Sca-1$^+$ CD150$^+$CD48$^-$) gradually decreases in accordance with development in the tissues such as fetal liver, newborn liver, and 2 week-old BM [10]. Conditional deletion of Sox17 in hematopoietic cells in neonates results in a significant decrease in the HSC number in BM, whereas Sox17-deletion in the adults causes no significant reduction of HSC number [11]. These results indicate the importance of Sox17 in HSC development and/or maintenance of HSCs in the fetus and neonate, but not in the adult.

We recently reported that forced expression of *Sox17* gene in CD45^{low}c-Kit^{high} AGM cells, which are the major hematopoietic component of IAHCs, inhibited differentiation of HSCs/hematopoietic progenitor cells (HPCs) and led to repetitive formation of cell clusters during several passages of the coculture with stromal cells in the presence of SCF, IL-3, and TPO [12]. Intra-BM transplantation of Sox17-transduced cells into lethally irradiated mice revealed that Sox17-transduced cells have long-term repopulating ability *in vivo*. During coculture with stromal cells, shutdown of the expression of the transduced *Sox17* gene immediately induced hematopoietic differentiation. These results indicate that Sox17 contributes to the maintenance of cell clusters containing HSCs in the midgestation AGM region and plays a pivotal role in controlling the HSC fate decision between self-renewal and differentiation in fetal hematopoiesis [12]. Based on the observation that Sox17-transduced cells are not able to form the cell cluster in the coculture with stromal cells without the stimulation of these cytokines, in the present study, we examined the role of each of the three cytokines, SCF, IL-3, and TPO, in the formation and the maintenance of the Sox17-transduced hematopoietic cell clusters.

2. Materials and methods

2.1. Animals

ICR and C57BL/6 mice were purchased from Japan SLC. Animal experiments were performed in accordance with institutional guidelines approved by the Animal Care Committee of Tokyo Medical and Dental University.

2.2. Isolation of CD45^{low}c-Kit^{high} cells from E10.5 AGM regions

The E10.5 AGM regions excised from ICR mice 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 washed with HBSS containing 10% (v/v) FCS and 250 μg/ml DNase I. The washed cells were 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). 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 FACSAriaII (Becton Dickinson, Lincoln, NJ, USA). The results of flow cytometry were analyzed with FlowJo (Three Star Inc., Ashland, VT, USA). Sorted CD45^{low}c-Kit^{high} cells were seeded on OP9 stromal cells in α -minimal essential medium (α -MEM) supplemented with 10% (v/v) FCS, 10 ng/ml IL-3, 50 ng/ml SCF, and 10 ng/ml TPO.

2.3. Overexpression of Sox17 in CD45^{low}c-Kit^{high} cells prepared from E10.5 AGM regions

Sox17 was inserted into the pMY-internal ribosomal entry sequence (IRES)-green fluorescence protein (GFP) vector with a Flag-tag [13]. Plat-E cells for packaging ectopic retroviruses [14] were seeded 1 day prior to transfection. To obtain Sox17-IRES-GFP gene-encoding retroviruses, transfection of Plat-E cells with plasmid DNA was performed using Trans IT-293 Reagent (Mirus, Madison, WI, USA). FACS-sorted CD45^{low}c-Kit^{high} cells prepared from E10.5 AGM regions were infected with the retroviruses in the presence of 10 μg/ml polybrene. After 2.5 h, the medium was replaced with α-MEM supplemented with 10% (v/v) FCS, 10 ng/ml IL-3, 50 ng/ml SCF, and 10 ng/ml TPO. After 4 days of culture, the infected GFP$^+$ cells were sorted by FACSAriaII and reseeded onto new OP9 stromal cells to proliferate the Sox17-transduced cells. The process was repeated every 3 or 4 days.

To analyze the effect of each cytokine on the formation of Sox17-transduced hematopoietic cell clusters, Sox17-transduced cells (2.5×10^2) were cocultured with OP9 stromal cells in the stimulation with all possible combinations of IL-3, SCF, and TPO. After 4 days of the culture, the size of hematopoietic cell clusters and the number of hematopoietic cells clusters were examined.

To examine the role of the TPO/c-Mpl signaling pathway in cell clusters, anti-mouse/rat TPO antibody (3, 10, 30 μg/ml, R&D systems, Inc., Minneapolis, MN, USA) was incubated with Sox17-transduced cells in the presence of TPO (10 ng/ml) on the OP9 stromal cells. After 3 days of culture, the number of hematopoietic cell clusters was counted. Sox17-IRES-GFP retrovirus-infected CD45^{low}c-Kit^{high} cells from E10.5 AGM regions were cocultured on the OP9 stromal cells with TPO and an inhibitor of the TPO/c-Mpl signaling pathway, U0126 (an inhibitor of MEK; Cell Signaling Technology, Inc.), AG490 (an inhibitor of JAK2; Sigma), SB203538 (an inhibitor of p38 kinase; Calbiochem), or LY294002 (an inhibitor of PI3K; Sigma). After 4 days of culture, the numbers of hematopoietic cell clusters and flattened hematopoietic colonies in the Sox17-transduced cells were counted.

2.4. Quantitative and semi-quantitative RT-PCR

cDNA was synthesized from total RNA isolated from GFP$^+$ cells using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Relative mRNA expression of c-Mpl was analyzed by quantitative RT-PCR analysis using Power SYBR Green PCR Master Mix (Applied Bioscience). Triplicate samples were examined on the 7500 real-time PCR system (Applied Bioscience) and the data was assessed by the relative standard curve method. Expression values for c-Mpl gene were normalized to the levels of β-actin gene. To analyze the semi-quantitative RT-PCR, PCR amplification was performed using rTaq (TAKARA Bio Inc., Otsu, Japan) under the following conditions: 95 °C for 3 min, and then cycles of 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 1 min. The primer sets used were as follows: 5′-CGGTATGCCTACCGAGGAGAGAAG-3′, 5′-CGCGGGACACATTCTTCACCCAG-3′ (c-Mpl); and 5′-CAGCCTGGCTGGCTACGTACA-3′, 5′-CCAGGGTGTGATGGTGGGAA-3′ (β-actin).

2.5. Semisolid colony-forming assays

Sorted cells (1.0×10^3) were added in Methocult™ (M3434; StemCell Technologies, Vancouver, BC, Canada) and cultured at 37 °C for 7 days. Individual colonies were scored by the morphology.

2.6. Western blotting

After starvation with serum free α-MEM for 4 h, Sox17-transduced cells were stimulated with TPO (25 μg/ml) for 10 min. Stimulated cells were lysed with lysis buffer containing 0.5% Nonident-40, 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 5 μg/ml aprotinin, 2 mM Na_3VO_4, and 1 mM (p-amidino)phenylmethanesulfonyl fluoride. The lysates were separated by