Studies on Extrinsic Regulation of Mouse Hematopoietic Cell Clusters during Early Embryogenesis

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Regulation of hematopoietic cell clusters in the placental niche through SCF/c-Kit signaling in embryonic mouse

Introduction

During mouse embryogenesis, hematopoiesis begins in the yolk sac (YS), producing mainly primitive erythroid cells at embryonic days (E) 7.5. Shortly thereafter, definitive myelo-erythroid progenitor cells appear in the YS, which seed the fetal liver. This process, termed primitive hematopoiesis, diminishes at E12.5, when definitive hematopoiesis, which sustains the adult blood system through hematopoietic stem cells (HSCs), begins in fetal liver. Although there is controversy over where HSCs are generated—in the extra-embryonic YS or intra-embryonic para-aortic-splanchnopleural mesoderm (P-Sp)/Aorta-Gonad-Mesonephros (AGM) region—recent studies suggest that both the YS and P-Sp/AGM region contain HSCs capable of reconstituting adult bone marrow hematopoiesis. Thereafter, these AGM HSCs are thought to circulate and colonize fetal liver, where HSC expansion occurs. In addition to these sites, several reports suggest that the placenta functions not only in gas exchange and fetal nutrition but also in hematopoiesis at approximately mid-gestation. It is also reported that a significant proportion of hematopoietic progenitor cells (HPCs), including highly proliferative potential colony forming cells (HPP-CFCs), are located in the mouse placenta. HSCs are detected at this site by E11.5 and the number of long-term

reconstituting (LTR)-HSCs dramatically increases from E11.5 to E12.5, resulting in a 15-fold increase in HSC activity compared with that of the AGM region. Taken together, these findings indicate that mouse placenta is likely a site for HSC generation and expansion at mid-gestation. HSCs are regulated by intrinsic programming and by extrinsic signaling from so-called niche cells. However, it is unclear how HSC generation and expansion is regulated in the placenta.

Results

To determine their compartment of origin and define extrinsic signals governing their commitment to this lineage, I identified hematopoietic cell (HC) clusters in mouse placenta, defined as cells expressing the embryonic HSC markers CD31/CD34/c-Kit, by immunohistochemistry. To determine how clusters are extrinsically regulated, I isolated niche cells using laser capture micro-dissection and assayed them for expression of genes encoding hematopoietic cytokines. Among a panel of candidates assayed, only *Stem Cell Factor* (*SCF*) was expressed in niche cells. To define niche cells, endothelial and mesenchymal cells were sorted by flow cytometry from dissociated placenta and hematopoietic cytokine gene expression was investigated. The endothelial cell compartment predominantly expressed SCF mRNA and protein. To determine whether SCF/c-Kit signaling regulates placental HC cluster proliferation, I injected anti c-Kit neutralizing antibody into E10.25 embryos and assayed cultured embryos for expression of hematopoietic transcription factors. *Runx-1*, *c-Myb* and *Gata2* were down-regulated in the placental HC cluster fraction relative to controls. These observations demonstrate that placental HC clusters originate from the allantois and are regulated by endothelial niche cells through SCF/c-Kit signaling.

Discussion

To examine mechanisms governing niche cell regulation of HSCs in the mouse placenta, it was necessary to gain insights into their cellular interactions through observation of their morphology and evaluating cells based on marker expression. Previous studies characterized placental HSCs primarily by flow cytometry, cell culture and transplantation, while immunohistochemical analysis of HC clusters has not been extensively undertaken. I successfully identified c-Kit^{+}/CD31^{+}/CD34^{+} HC clusters in the mouse placenta and AGM region. Clusters in the placenta were attached to endothelial cells, as has been observed in the AGM region, a site of HSC generation, suggesting that the placenta might be a site for HSC generation.

HSCs are regulated by niche cells surrounding HSCs. However, it remains unclear how embryonic HSCs are regulated by niche cells. In the bone marrow, expression of niche cell markers such as N-cadherin and CXCL12 enables their isolation by flow cytometry and has contributed greatly to an understanding of niche regulation. Conversely, investigation of the placental niche has been impeded by a lack of markers for placental niche cells. To address this issue, I isolated niche cells surrounding HC clusters in placenta by LCM. Using this system, I obtained niche cells despite the lack of markers. HC clusters were found inside of blood vessels, suggesting that niche cells are mostly composed of endothelial cells. In addition, I sorted out both endothelial and mesenchymal cells, and performed real-time PCR with *SCF* gene. The gene expression

analysis revealed that *SCF* is predominantly expressed in niche cells, and protein expression analysis suggested that SCF is predominantly expressed in niche endothelial cells. To understand the role of the SCF/c-Kit signal in regulating placental HSCs, I performed a loss-of-function experiment *in vivo* to inhibit SCF/c-Kit signaling in the mouse placenta by utilizing a WEC system with E10.25 embryos – a stage suitable for manipulation. SCL is not required for HSC development once commitment to hematopoietic lineages has occurred. However, Gata-2 is critical for definitive hematopoiesis and functions in the generation and expansion of HSCs in the AGM region. My study confirmed that expression of *Runx-1*, *c-Myb* and *Gata-2* was significantly down-regulated compared to control samples in c-Kit loss-of-function analyses but *SCL* expression was not altered. c-Kit receptor activation plays a major role in regulating survival, proliferation and self-renewal of HSC phenotypes, but how SCF/c-Kit signal regulates *Runx-1*, *c-Myb* and *Gata-2* remains unclear. In addition to SCF/c-Kit signaling, other signals may regulate HC clusters.

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Mesonephros regulates myeloid differentiation of hematopoietic cell clusters in the aorta-gonad-mesonephros region through Csf1/Csf1r signaling.

Introduction

During mouse embryogenesis, there are two distinct waves of hematopoietic emergence: a primary hematopoiesis, giving rise to transient populations of progenitors that differentiate into erythrocytes and macrophages, and a definitive hematopoiesis, sustaining the adult type blood system through hematopoietic stem cells (HSCs) capable of reconstituting adult bone marrow hematopoiesis. HSCs are detectable in the para-aortic-splanchnopleural mesoderm (P-Sp)/Aorta-Gonad-Mesonephros (AGM) region at embryonic day (E) 10.5 where cell aggregates, hematopoietic cell (HC) clusters are observed. HSCs are considered to reside in these HC clusters based on the observation of *Runx1*$^{-/-}$ embryos that lack neither HC clusters nor HSC activity. Extrinsic factors reportedly play roles in regulating AGM HSCs. Extrinsic regulation by niche cells is pivotal for HSC regulation in the AGM region. Intereukin-3 within the lumen of the aorta increases the number of HSCs in the AGM region. It is also reported that SCF/Kit signal is involved in HC cluster formation in the AGM region. However, extrinsic regulation of HC cluster differentiation is largely unknown.

Result

To investigate how HC clusters are extrinsically regulated by mesonephros, I employed Sall-1 GFP Tg mouse embryos that enable me to identify mesonephros expressing *Sall-1* gene. Transverse section of the AGM region revealed that mesonephros expresses GFP. Sorted mesonephric cells at E10.5 were examined by real-time PCR and exhibited the highest *Csf1* gene expression among several hematopoietic cytokine genes, and expressed Csf1 protein by immunocytochemistry. Csf1r, a receptor for Csf1 was expressed on some HC clusters by immunohistochemistry and flow cytometry. Csf1r(+) HC clusters expressed *Cebpa* gene higher than Csf1r(–) HC clusters, suggesting Csf1r(+) HC clusters possess higher myeloid-potential than Csf1r(–) HC clusters. Colony formation assay of HC clusters with Csf1 showed increased number of myeloid colonies in response to Csf1 signal through Csf1r. Furthermore, immunohistochemistry revealed HC clusters expressed Cleaved Caspase-3, a down-stream signal of Csf1/Csf1r. Taken together, the findings demonstrate that Csf1 secreted from mesonephros accelerates myeloid differentiation of HC clusters in the AGM region likely through Cleaved Caspase-3.

Discussion

Sall-1 is expressed abundantly in embryonic kidney including mesonephros. It was reported that using Sall-1 GFP Tg mice, GFP positive cells from embryonic kidneys of these mice were isolated by flow cytometry to identify genes expressed in the developing kidney. I also employed the same strategy to identify hematopoietic cytokine genes secreted from mesonephros in the AGM region. Gene expression of multiple hematopoietic cytokines in mesonephros was highest at E10.5. This observation implys that mesonephros contributes to HC cluster regulation by cytokine production at E10.5. The highest gene expression of evaluated cytokines was *Csf1* mRNA. The effects of Csf1 are mediated by a high-affinity receptor Csf1r. I confirmed protein expression of both Csf1 in mesonephric cells and Csf1r on HC clusters, suggesting that HC clusters are likely regulated by Csf1 secreted from mesonephros.

Cebpa mRNA expression in Csf1r+/CD31+/CD34+/c-Kit+ cells was significantly higher than that in Csf1r-/CD31+/CD34+/c-Kit+ cells. Cebpa is a transcription factor that plays a critical role in myelopoiesis. Expression level of Cebpa is specifically upregulated with myeloid differentiation. Collectively, Csf1/Csf1r signaling likely plays a role in myeloid commitment of HC clusters during their maturation. Colony assay demonstrated the increased myeloid potential in the presence of Csf1. Specifically, CFU-M and CFU-GM were significantly increased, suggesting that Csf1/Csf1r signaling contributes to the differentiation of HC clusters in the AGM region.

To identify downstream signaling of Csf1/Csf1r on HC clusters, I employed an

intracellular signaling assay, which enabled me to detect 18 important and well-known signaling molecules when phosphorylated or cleaved. Analysis indicated the greatest fold increase of Cleaved Caspase-3 in AGM cells in the presence of Csf1. Cleaved Caspase-3 was predominantly expressed in HC clusters. Although Cleaved Caspase-3 is a marker of apoptosis, recent studies clarified its role for regulating differentiation of HSCs. Collectively, these findings suggest that Csf1 secreted from mesonephros accelerates HC cluster myeloid differentiation in the AGM region, possibly via Caspase-3 cleavage.