Characterization of purified intraembryonic hematopoietic stem cells as a tool to define their site of origin

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Little is known about hematopoietic stem cell (HSC) development from mesoderm. To gain more information on the intraembryonic HSC site of origin, we purified multipotent hematopoietic progenitors from the aorta–gonads–mesonephros (AGM) of mice. This population, expressing c-Kit, AA4.1, CD31, and CD41, but not Flk1, and mainly negative for CD45, proved capable of long-term reconstitution in sublethally irradiated Rag2 γ c^{-/-} recipients. We assigned the expression of GATA-2, GATA-3, and lmo2 to AGM-HSC, whereas erythromyeloid progenitors express only GATA-2. This unique combination of surface markers and transcription factors could be allocated in the AGM to the intraaortic clusters and the subaortic patches underlying aortic endothelial cells. Taken together, those data indicate that embryonic HSCs (*i*) differ from their fetal liver and adult counterpart by the low expression of CD45, (*ii*) do not colocalize with aortic endothelial cells as previously thought, and (*iii*) are localized, at 10.5 days postcoitum, in the splanchnic mesoderm underlying aortic endothelial cells, within GATA-3⁺CD31⁺ cell clusters.

aorta–gonads–mesonephros | yolk sac

During mouse development, the first hematopoietic cells, consisting of precursors with erythroid and/or myeloid potential (1), appear in the yolk sac (YS) blood islands, from 7 days postcoitum (dpc). Before the establishment of circulation (8 dpc), the first progenitors endowed with long-term reconstitution (LTR) activity are generated in the intraembryonic splanchnopleura and are absent from the YS (2, 3). After circulation is established, multipotent precursors with LTR activity (4, 5) can be found in the blood (6) and in the YS (7). After 10 dpc the paraaortic splanchnopleura, which now comprises the aorta, gonads, and mesonephros, is called AGM. Hematopoietic precursors, present in the AGM after 10.5 dpc, have LTR capacity when transferred in adult normal recipient mice (4).

It was previously suggested that the floor of the aorta and/or the underlying splanchnic mesoderm is the site of origin of intraembryonic hematopoietic stem cells (HSCs) (8–12). Clusters of basophilic cells located within the floor of the aorta were detected from 10 to 10.5 dpc, further characterized as hematopoietic cells, and taken as a sign of ongoing hemogenesis. These hematopoietic intraaortic clusters (HIACs) have been described in all vertebrate embryos, including humans (for review, see ref. 13). Other structures potentially involved in intraembryonic HSC generation are the subaortic patches (SAPs), located below the aortic floor, which express the transcription factor GATA-3 (14) and the AA4.1 antigen (15). These SAPs are also present in human embryos (16, 17). In contrast to HIACs, which are detected only at the peak of intraembryonic HSC production (8), the SAPs are present in the intraembryonic hemogenic site for the duration of HSC generation. An involvement of both SAPs and HIACs in HSC generation is strengthened by the fact that

both structures disappear (11, 14) concomitantly with the cessation of AGM-HSC production at 12 dpc (8).

The understanding of the process leading to intraembryonic HSC generation is currently a matter of debate that can be clarified by precisely locating the structure where this generation occurs. In the avian and murine models as well as in human embryos, AGM-hematopoietic activity has been shown to derive from cells harboring markers shared by endothelial and hematopoietic lineages, which also lack the panhematopoietic marker CD45 (18–20). It has thus been proposed that the endothelium of the aortic floor displays “hemogenic” activity and is able to give rise to HSCs.

Here we correlated surface phenotype, *in vitro* differentiation potential, LTR activity, and gene expression of 10.5-dpc AGM populations. We show that CD45⁺ cells contain only macrophage precursors. CD45⁻/c-Kit⁺AA4.1⁺ cells from either AGM or YS are differentially enriched for two distinct types of progenitors. Erythromyeloid precursors are found at a frequency of 1:2 in YS. Multipotent precursors are present at frequencies higher than 1:3 in AGM, representing more than 80% of total multipotent cells in this site. LTR activity in alymphoid recipients was strictly ascribed to CD45⁻/c-Kit⁺AA4.1⁺ AGM cells that can therefore be considered as HSCs.

HSCs purified according to these criteria express a number of cell surface markers (CD31, c-Kit, and AA4.1) and transcription factors (lmo2 and GATA-2) common to cells of both hematopoietic and endothelial lineages. However, they may be distinguished from endothelial cells (ECs) by the lack of Flk1 and the expression of CD41 (21–23) and GATA-3. The phenotype and gene expression profile of purified HSCs were compared with those obtained by *in situ* hybridization and immunostaining of the AGM (ref. 14 and present work). Coexpression of GATA-2, GATA-3, lmo2, CD41, and CD31 was observed exclusively in two distinct anatomical structures, the HIAC and the SAP (14). We propose that HSCs are generated in the SAP before they form the aortic clusters and before they enter blood circulation.

Materials and Methods

Animals and Dissections. Two C57BL/6 congenic lines (H2^b) bearing the Ly5.1 and Ly5.2 alleles of the CD45 marker and their F₁ progeny were used in this study. Rag2 γ c^{-/-} B10BR mice (H2^k) were used as recipients for LTR assays. Embryonic development was estimated by considering the day of vaginal

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Abbreviations: dpc, days postcoitum; AGM, aorta–gonads–mesonephros; YS, yolk sac; HSC, hematopoietic stem cell; EC, endothelial cell; HIAC, hematopoietic intraaortic cluster; SAP, subaortic patch; LTR, long-term reconstitution.

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Fig. 3. Localization of AGM hematopoietic subsets in 10- to 10.5-dpc (30–35S) embryos. Confocal expression analyses (A) allow one to discriminate between macrophages (CD45⁺CD31⁺CD41[−], arrowheads) and HSCs (CD41⁺CD31⁺CD45^{−/lo}, arrows). HSCs are located within the intraaortic clusters (stars) and within the SAPs (asterisks). (B) CD31 is expressed by ECs and by GATA-3⁺ SAPs (asterisk). (Bars, 50 μ m.)

AGM fraction E is the expression of L-plastin (31) and low levels of CD45 mRNA (Fig. 2C).

We show that, at this stage of development, HSCs express c-Kit, AA4.1, CD41, and CD31, whereas expression of Flk1 is undetectable. Flk1⁺CD31⁺ cells that express variable levels of c-Kit and AA4.1 do not generate a hematopoietic progeny and according to this expression pattern likely correspond to ECs.

At 10.5 dpc, Fraction E Cells Are Localized Within Intraaortic Clusters and SAPs. We next analyzed by RT-PCR the expression, within fraction E, of genes shared by hematopoietic cells and ECs [GATA-2 (32) and *lmo2* (14)] and genes not expressed by ECs [GATA-3 (33)]. Fig. 2B shows that whereas GATA-2, GATA-3, and *lmo2* are highly expressed in AGM fraction E, only GATA-2 is expressed at a notable level in the YS. Considering that HSCs are mostly represented in AGM fraction E and that erythromyeloid precursors are predominant in the YS, we conclude that erythro-

myeloid precursors are likely to express only high levels of GATA-2, whereas HSCs express GATA-2, GATA-3, and *lmo2*.

To locate in the AGM the various subpopulations characterized by flow cytometry, *in vitro* and *in vivo* potential assays, and gene expression analysis, we performed *in situ* hybridization (GATA-2 and GATA-3) and multiple immunostainings (CD41, CD31, and CD45) on 10- to 10.5-dpc (30–40S) AGM sections to discriminate between the various fractions.

Fraction A cells, corresponding to macrophages, were identified by their CD45⁺CD31⁺CD41⁻ phenotype (Fig. 1). CD45⁺ cells were found surrounding the aorta (Figs. 3A and 4C).

CD41⁺CD31⁺CD45^{-lo} cells, which correspond to HSCs, were present only in the ventral side of the AGM, either in SAPs below the aortic floor (Figs. 3 and 4; see also Fig. 8, which is published as supporting information on the PNAS web site) or within the HIAC (Figs. 3 and 8). This labeling pattern colocalizes with GATA-3 expression (Fig. 3). At later stages, when HSCs are more numerous

Fig. 4. Localization of AGM-HSCs and their environment in 10.5-dpc (35–40S) embryos. From 10.5 dpc (35–40S), the subendothelial CD31⁺GATA-3⁺ layer evolves into more compact SAPs (asterisks), which express GATA-2 mRNA (A) in addition to GATA-3 (B). Confocal analyses (C and D) show that HSCs are located within the SAPs. CD45⁺ macrophages (arrowheads) do not express CD41. XZ projection of the image stack (D) that comprises the top section in C shows that CD31⁺CD41⁺ HSCs are present only in the ventral part of the AGM (below the straight line joining the cardinal veins (CV)). Their distribution forms a continuum from the SAPs (asterisks) to the aortic floor. Note that CD31⁺CD41⁺ HSCs constitute only a minority of the CD31⁺GATA-3⁺ SAP cells. Stainings in A–C were performed on alternate sections of the same embryo. (Bar, 50 μ m.)

Several lines of evidence point to the SAP, rather than the HIAC or aortic floor, as the primary site of origin of intraembryonic HSCs in the embryo:

- (i) The GATA-3⁺ SAP become detectable concomitantly with the production of the first intraembryonic precursors (8.5–9 dpc) (7), whereas HIACs appear only at the AGM stage (10 dpc on) (11), when the number of precursors generated has significantly increased (8) (Fig. 5B).
- (ii) Lateral examination of confocal sections (Fig. 4D) shows that HSCs form a continuum between the SAP and the HIAC, suggesting that these cells migrate from one site to the other. The most likely direction of migration appears to be from SAP to HIAC and finally into the aortic blood flow, because it contains a significant number of multipotent precursors at that stage (6). This model nevertheless requires *in vivo* cell tracking experiments to be confirmed.
- (iii) The combinatorial approach undertaken here allowed us to distinguish between ECs and hematopoietic cells, by GATA-3, CD41, and Flk1 differential expression. The 3D examination of SAP anatomy also distinguishes SAP cells from ECs because the CD31⁺ cell clusters located below the aortic floor do not harbor a lumen and do not connect with the vascular network (Fig. 9).

Recent reports suggesting that the aortic floor in 11-dpc AGM (18, 20) could be the source of HSCs were based on the fact that CD31⁺CD45[−] cells, considered as ECs, display a hematopoietic activity. Through the use of additional markers and colocalization analysis, we here show that HSCs and aortic floor ECs are not overlapping populations.

We thus propose that HSCs are generated in the SAP, migrate toward the ventral region of the aorta, and protrude through the endothelium, contributing to the formation of the HIAC and leading to their release into circulation, from where they colonize the fetal liver (Fig. 5C).

In the course of our colocalization studies, we encountered only a limited number of such CD31⁺CD41⁺CD45^{−/lo} cells in 10.5-dpc embryo sections, a result that concurs with the number of HSCs, previously estimated at ≈80–100 per embryo (8). It thus appears that the relative size of cells expressing GATA-3 and/or CD31 in the SAP greatly exceeds the number of HSCs present in the embryo. SAPs may constitute the environment suitable for the emergence of intraembryonic HSCs. Our results show that SAPs, which can be characterized by the expression of CD31 and AA4.1 and lack of CD45, contain HSCs that also express CD41. Further characterization of the SAP, now made possible by this observation, will be necessary to understand the relationship of SAP cells and native HSCs and to assess whether the SAP modulates HSC generation and self-renewal.

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