Aorta-Associated CD34+ Hematopoietic Cells in the Early Human Embryo

By Manuela Tavian, Laure Coulombel, Dominique Luton, Hélène San Clemente, Françoise Dieterlen-Liévre, and Bruno Peault

Hematopoiesis is established from circulating blood stem cells that seed the embryonic rudiments of blood-forming tissues, a basic notion in developmental hematology. However, the assumption that these stem cells originate from the extraembryonic mesoderm, where primitive hematopoiesis is initiated by intrinsic precursors, has been reconsidered after analysis of blood cell development in avian embryo chimeras: yolk-sac-derived stem cells do not contribute significantly to the definitive blood system, whose first forerunners develop independently along the ventral aspect of the embryonic aorta. Recently, the homologous intraembryonic tissues of the mouse have been submitted to sensitive in vivo and in vitro assays, which showed that they also harbor multipotential hematopoietic stem cells. We have now identified a dense population of hematogenous cells, marked by the surface expression of the CD34 glycoprotein, associated with the ventral endothelium of the aorta in the 5-week human embryo. Therefore, we extend to the human species the growing evidence that intraembryonic hematopoietic cells developing independently of the yolk sac might be the real stem of the whole blood system.

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MATERIALS AND METHODS

Human tissues. Fourteen human embryos at 23 to 50 days of development (ie, postconception) were obtained immediately after voluntary terminations of pregnancy induced by administration of the antiprogestosterone compound Mifepristone. In all cases, informed consent to the use of the embryo in research was obtained from the patient, and embryos were collected according to the guidelines and with the authorization of the French Comité National d'Ethique. Gestational age was estimated on developmental anatomical criteria.

Antibodies and lectin. Monoclonal antibodies to CD34 (HPCA-1 and phycoerythrin [PE]-HPCA-2), CD45 (HLE-1), and CD38 directly coupled to fluorescein isothiocyanate were purchased from Becton Dickinson (San Jose, CA) and that to CD31 (JC70A) from DAKO (Glostrup, Denmark). Biotinylated Ulex europaeus agglutinin I was supplied by Vector (Burlingame, CA).

Tissue processing and section staining. Embryos fixed overnight at 4°C in phosphate-buffered saline (PBS), 4% paraformaldehyde.

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(vol/vol) were rinsed in PBS, dehydrated, and included in paraffin. Five-micron-thick sections were immersed three times for 7 minutes in toluene, absolute ethanol and finally 95% ethanol. Endogenous peroxidases were inhibited for 20 minutes in methanol containing 0.2% hydrogen peroxide. Sections were then washed with PBS 0.25% Triton X-100 (Sigma, St Louis, MO). The primary antibody or lectin was added overnight at 4°C. After washing with PBS-Triton X-100, incubation was performed for 1 hour at room temperature with, first, biotinylated rabbit-antimouse Ig antibody (DAKO) and subsequently with peroxidase-labeled streptavidin (DAKO). Peroxidase activity was shown with 0.025% (vol/vol) 3,3'-diaminobenzidine (Sigma) in PBS containing 0.015% hydrogen peroxide. Slides were counterstained with Harris’ hematoxylin and mounted in aqueous medium (BioGenex Laboratories, Dublin, CA) for examination on an Optiphot-2 microscope (Nikon, New York, NY).

Three-dimensional reconstruction and visualization of serial immunostained tissue sections. Tissue sections were recorded with a CDD black and white video camera (Cohu Inc., San Diego, CA) adapted to a Leitz Laborlux S microscope (Weitzlar, Germany) and tissue contours were acquired on a Deskpro 386/25 PC computer (Compaq Computer Co, Houston, TX), using the HISTO software (Biocom, Les Ulis, France). Printed contours were then transferred to an SD-420E graphic table (WACOM Technology Co, Vancouver, WA) connected with a Quadra 950 Macintosh computer (Apple Co, Cupertino, CA), using the CANVAS 3.0 software (Deneba Software, Miami, FL). Finally, serial contours were treated with the 3D TURBORENDER 6.0.6 software (SOFTX, Plaisir, France) to create a three-dimensional orientable model of the tissue and translate it into a synthetic image.

In vitro hematopoietic cell assays. The trunk area containing the segment of the aorta in which we detected accumulated CD34+ cells (see Results) was selectively removed from three human embryos, two at 30 to 35 days and one at 35 to 40 days of gestation, and its hematopoietic potential assessed in vitro. Care was taken to exclude the liver rudiment that was assayed separately. The heart and limbs were dissociated gently during the dissection was also plated. Tissues were dissociated gently by pipetting, but no attempt was made to obtain single cell suspensions. Tissue fragments were immediately seeded in 96-well plates precoated with a confluent layer of MS-5 murine stromal cells. Cultures were performed in long-term culture medium (12.5% FCS [Techgen, Les Ulis, France], 12.5% horse serum [Hyclone Laboratories, Logan, UT], 10−4 M 2-Mercaptoethanol in a minimum essential medium [αMEM], at 37°C and medium was changed twice weekly. No exogenous cytokines were added. At time intervals non-adherent and adherent cells were obtained and pooled, counted, and processed for phenotypic analysis by flow cytometry and colony assays. Colony assays were performed in methocelulose as previously described, in the presence of recombinant human (rh) cytokines including rh erythropoietin (Epo, 21 U/mL), rh stem cell factor (50 ng/mL), rh interleukin-3 (100 U/mL), and rh granulocyte-colony stimulating factor (10 ng/mL). The three latter cytokines were kindly provided by Amgen (Thousand Oaks, CA), Sandoz (Basel, Switzerland), and R. Belton Laboratories (Neuilly sur Seine, France), respectively. Colonies were scored at least twice at days 10 and 15.

Cells produced during the coculture were also analyzed by flow cytometry for their expression of the CD34 and CD38 antigens on a FACSort (Becton Dickinson) equipped with the Cellquest software (Becton Dickinson).

RESULTS

We observed that CD34 expression accompanies the early ontogeny of the human vascular system, because we detected it on endothelial cells in the yolk sac and embryo at 23 days of gestation, the earliest stage tested (not shown). This is in agreement with the recent description of murine CD34 anti-gen emergence on endothelial and hematopoietic cells in the early developing yolk sac. At 35 days of gestation, CD34 was uniformly expressed at the luminal aspect of endothelial cells in developing intraembryonic blood vessels (Fig 1A). Strikingly, a CD34-labeled thickening of the ventral aspect of the dorsal aorta was also observed in the preumbilical region of the embryo at that stage, and seen at a higher magnification to be composed of packed, CD34−, round cells in close apposition to the endothelium (Fig 1B). Similar intraaortic CD34+ cell clusters were observed in six different human embryos ranging from 30 to 37 days of gestation. In each case, these clusters were precisely localized in the preumbilical region, at about the level of the anterior limb bud, but more rostral or more caudal sections of the aorta did not contain them (Fig 1C). Three younger (28 to 30 days), another 30 to 34 days, and a 7-week human embryo were found to be devoid of endothelium-associated aggregated CD34+ cells.

Further phenotypic characterization was performed to document the relationship between aortic endothelium and the locally associated CD34+ cells. The lectin Ulex europaeus, which specifically binds to adult vascular endothelial cells, marked most of the cells of the endothelial lining of the 35-day human dorsal aorta, but did not show affinity for the above-described intraaortic cell clusters (Fig 2A). A reverse pattern was observed after immunostaining of the

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Fig 1. CD34 expression in the 25-day human embryo. (A) Low magnification of an immunostained transverse section in the immediate preumbilical region shows CD34 expression by the endothelial cells lining the dorsal aorta (Ao), the blood vessels (CV: cardinal vein), and capillaries present around the neural tube (NT) and mesonephric rudiment (Mn) (original magnification [OM] × 92). (B) Magnified view of the aorta on the same section shows the ventral thickening of the vessel wall resulting from the accumulation of round CD34+ cells. Nucleated erythrocytes are present inside the lumen (OM × 370). (C) In a more caudal region of the same embryo, no CD34-expressing cells are seen associated with the wall of the aorta (OM × 370).

Fig 2. The CD34-expressing cells present on the floor of the 5-week human embryonic aorta are nonendothelial hematopoietic cells. (A) The endothelium-specific lectin Ulex europaeus binds to aortic endothelial cells, but not to the CD34+ cells clumped on the vessel floor. (B) CD31 is expressed on both endothelial cells and adherent intraaortic cells; the latter also express the pan-leukocyte CD45 molecule (C), which is expectedly absent from vascular endothelium. (A and B) Transverse sections from the embryo pictured in Fig 1. (C) Transverse section in the same region of another embryo at the same stage of development (OM × 370).

Fig 3. Computer-assisted reconstruction of the dorsal aorta from a 5-week human embryo. Seventy-two serial 5-μm sections made in the preumbilical region (framed on the picture of the whole embryo on the left) were immunostained for CD34. The CD34+ intraaortic hematopoietic cells are in green color. Ao, dorsal aorta; AL, anterior limb bud; L, liver; H, heart; YS, yolk sac.
Table 1. Hematopoietic Progenitor Cells Produced by Different Human Embryo Body Parts Cocultured With MS-5 Cells

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Absolute No. of Progenitor Cells Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp 1</td>
</tr>
<tr>
<td>Preumbilical aortic region</td>
<td>2000</td>
</tr>
<tr>
<td>Liver</td>
<td>350</td>
</tr>
<tr>
<td>Heart</td>
<td>0</td>
</tr>
<tr>
<td>Limbs</td>
<td>5</td>
</tr>
<tr>
<td>Blood</td>
<td>ND</td>
</tr>
<tr>
<td>Umbilical cord</td>
<td>40</td>
</tr>
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Body parts were dissected from three different human embryos at 35 to 40 days (Exp 1) or 30 to 35 days (Exp 2 and 3) and cocultured with MS-5 murine stromal cells as described (see Materials and Methods). To assess the number of progenitor cells produced, the content of each coculture (adherent and nonadherent cells) was plated in standard methylcellulose colony assays after either 4 (Exp 3) or 10 to 12 (Exp 1 and 2) days in coculture with MS-5. Numbers refer to the absolute number of clonogenic progenitor cells generated by the distinct embryonic tissues tested.

The production of clonogenic progenitors in the human yolk sac and embryonic blood and liver.26 Among other indirect arguments, a sharp decrease in erythroid burst-forming unit frequency in the yolk sac at 5 weeks was paralleled with the

DISCUSSION

The CD34+ hematopoietic cells seen here, accumulated within the human embryonic aorta, exhibit a similar anatomic localization as the intraembryonic stem cells that arise from the ventral wall of the avian aorta at an equivalent developmental stage, express the MB1/QH1 marker of hematopoietic and endothelial cells,24 and most likely generate both clonogenic progenitors of both granulocytic and erythroid lineages in a short-term stroma-dependent culture assay. Lack of similar activity in wells seeded with cells from circulating blood supports the idea that functional hematopoietic sites exist in the vicinity of the preumbilical truncal segment of the human embryonic aorta.
dramatic increase of those progenitors from week 4.5 in the liver anlage to suggest a monoclonal hypothesis of human blood cell development, according to which yolk-sac-derived stem cells would egress to the liver. Such a route of migration was not, of course, shown experimentally nor has it ever been described in more easily manipulated animal embryos, although the strict dependency of liver hematopoietic development upon extrinsic stem cell seeding has been firmly established.27

The limited availability of viable human tissues at those early developmental stages, the scarcity of appropriate and sensitive assay systems for the earliest human hematopoietic stem cells, as well as the considerable technical difficulties met to selectively dissect that minute cell cluster out of the 10-mm long 5-week human embryo, will delay the direct functional assessment of those aorta-associated CD34+ cells. However, three preliminary distinct culture experiments have already clearly shown that the area of the 30- to 40-day embryonic trunk that includes the cluster-bearing aortic segment generates much higher numbers of CD34+ cells and clonogenic progenitors than any other part of the embryo, including the liver rudiment. These data are in agreement with the results of a separate study performed by some of us, which showed that the number of both erythroid and granulocytic progenitor cells detected in colony assays established with cells freshly isolated from the eviscerated embryo body equalled or exceeded that found in the same assays established with cells from the liver rudiment (Huyhn et al, in press). Of note, the extensive hematopoietic potential of the dorsal aorta was best detected after a 10-day exposure of that territory to the inductive influence of BM stromal cells. The delay required before CD34+/progenitor cell proliferation was detected and the fact that both granulocytic and erythroid colonies were present suggest, but do not prove, that clonogenic progenitors arose from the proliferation of small numbers of primitive multipotent progenitor cells.

Interestingly, it appears from our experience of CD34 detection in embryonic, fetal, and adult human tissues that the intraaortic cell clusters described herein represent the densest local accumulation of hematopoietic CD34+ cells encountered throughout the development of the human blood system. Even at the crucial phases of hematopoietic development in the yolk sac and embryonic liver, CD34+ hematopoietic cells remain rare and scattered in those blood-forming tissues; they are even absent from the early BM anlage (Charbord et al, manuscript submitted for publication). A molecular analysis of the adhesion and regulation factors expressed in that unique cell population is underway, which may provide new insights into the biology of human blood stem cells.

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