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 hematogenic 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 anatomic criteria.

Antibodies and lectin. Monoclonal antibodies to CD34 (HPCA-1 and phycoerythrin [PE]-HPCA-2), CD45 (HL-1), and CD38 directly coupled to fluorescein isothiocyanate were purchased from Becton Dickinson (San Jose, CA) and to CD51 (JC/70A) 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 play the Thy-1 antigen at their surface. In addition, CD34 is expressed in the adult by vascular endothelial cells. Herein we describe an immunohistochemical analysis of 3- to 7-week human embryo sections that showed the existence of a spatially and temporally localized accumulation of hematopoietic CD34+ cells on the ventral wall of the aorta. The hematogenous potential of that territory is further suggested by its ability to generate high numbers of clonogenic hematopoietic progenitors on in vitro culture in the presence of BM stromal cells.

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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-streptavidin label (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 386/2S 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 tablet (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 (SOFT'X, 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 also isolated and similarly processed. The former was used as a source of circulating blood cells. In experiment 3, blood collected 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} mol/L 2-5-mercaptoethanol in a minimum essential medium [MEM], 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 methylcellulose 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. Bellon 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 antigen 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 rudiment, as 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 embryos 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...
both vascular endothelial cells and early hematopoietic progenitor cells were hematopoietic cells expressing surface molecules that mark cursor cells. Blood progenitor and stem cells. We have then used human dorsal aorta (Fig 3). CD34^+ nonendothelial in- termediate hematopoietic cell adhesion molecule-1 which is displayed at the surface of both vascular endothelial cells^22 and early hematopoietic pre- cursor cells.

Thus, the round adherent cells locally accumulated in the human dorsal aorta during the fifth week of gestation are hematopoietic cells expressing surface molecules that mark blood progenitor and stem cells. We have then used 72 serial, CD34-immunostained transverse embryo sections to computerize a spatial image of the preumbilical region of a 35-day human dorsal aorta (Fig 3). CD34^+, nonendothelial intra- arterial cells appeared to be densely gathered on the floor of the blood vessel and virtually absent from its dorsal aspect. Their number has been estimated at 831 cells on a distance spanning one to two somites in the immediate preumbilical area.

In recent attempts to correlate these descriptive findings with functional characteristics, three experiments were performed in which the preumbilical aortic region as well as other parts of the human embryo body were selectively dissected out, dissociated, and plated in short-term coculture (4 to 10 days) with MS-5 murine BM stromal cells, which are known to promote the development of human primitive progenitor cells. The production of clonogenic progenitors during that coculture period was used as an indicator of the hematopoietic activity of the various tissues analyzed. The rationale for prior short-term coculture comes from the results of pilot experiments that showed very low clonogenic activity when dissociated tissues were plated directly in methylcellulose. Results expressed in Table 1 unambiguously show that in all three samples tested, after 4 to 10 days in coculture, the preumbilical aortic region generated a high number of progenitor cells giving rise to large colonies in methylcellulose assays. A clearly higher number of clonogenic progenitors was recovered from the aortic region of the oldest embryo analyzed (35 to 40 days) than from the homologous area in the two other slightly younger samples tested (30 to 35 days). At least 30% to 50% of the colonies included erythroid cells. In agreement with these data, we also observed a high output of nonadherent round nucleated cells in the wells, more than 10% of which expressed the CD34 antigen as shown by flow cytometry (Fig 4). In contrast, the production of both clonogenic progenitors and non- adherent CD34^+ cells was strikingly lower in the wells seeded with liver cells dissected from the same embryos (Table 1 and Fig 4). Cocultures initiated with cells dissociated from the limbs or from other parts of the embryo (Table 1 and results not shown) did not generate significant numbers of hematopoietic cells or progenitors in these short-term cultures. These results show that at these stages of development, the aorta-associated tissues of the trunk contain high numbers of primitive precursor cells capable of producing 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.

### 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, and most likely generate all blood cell lineages but primitive erythrocytes. In the mouse, an embryonic territory comprising the aorta produces totipotent hematopoietic stem cells^23^, in agreement with functional data, endothelium-associated hematopoietic cell aggregates have been recently observed, in that species, in- side the omphalomesenteric artery, dorsal aorta, and umbilical artery around 10.5 days postconception, a developmental stage grossly equivalent to 5 weeks of human gestation. Thus, it is tempting to extrapolate that the intraaortic CD34^+ cells described herein are forerunners of the fetal and postna- tal human blood system. It will be important to determine whether the rare hematopoietic cells also detected in a suben- dothelial location, in the same area, prefigure the later development, like in the avian embryo, of paraaortic stem cell clusters. However, no evidence has been obtained yet from our preliminary observations that such structures develop in humans. Also remarkable is the simultaneousness of hepatic hematopoietic development as we could detect rare, scattered, nonendothelial CD45^- CD34^+ cells from the fifth week of gestation onward in the liver rudiment (not shown). Previous studies have shown the presence of pluripotent and more restricted hematopoietic progenitors in the human yolk sac and embryonic blood and liver. Among other indirect arguments, a sharp decrease in erythroid burst-forming unit frequency in the yolk sac at 5 weeks was paralleled with the

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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.

Table 1. Hematopoietic Progenitor Cells Produced by Different Human Embryo Body Parts Cocultured With MS-5 Cells

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Fig 4. CD34 expression by cells produced in vitro by the truncal aortic regions and liver rudiment dissected from 30- to 40-day human embryos. In two experiments (Exp 1 and Exp 2 from Table 1) the pre-umbilical aortic region was dissected out, dissociated mechanically, and cultured on a monolayer of MS-5 murine stromal cells. In Exp 1, the liver rudiment was similarly processed. After 8 to 10 days in coculture, nonadherent cells produced were labeled with the HPCA-2-PE antibody. CD34+ cell analysis was performed in the R1 gate excluding noncellular events and the proportion of R1 cells expressing the CD34 antigen is shown in R2. Light scatter properties of R2 CD34+ cells are indicated by the R3 gate.

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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