Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo

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SUMMARY

Hepatic hematopoiesis in the mouse embryo is preceded by two hematopoietic waves, one in the yolk sac, and the other in the paraaortic splanchnopleura, the presumptive aorta-gonad-mesonephros region that gives rise to prenatal and postnatal blood stem cells. An homologous intraembryonic site of stem cell emergence was previously identified at 5 weeks of human gestation, when hundreds of CD34^{++} Lin$^-$ high-proliferative potential hematopoietic cells border the aortic endothelium in the preumbilical region. In the present study, we have combined immunohistochemistry, semithin section histology, fluorescence-activated cell sorting and blood cell culture in an integrated study of incipient hematopoiesis in the human yolk sac, truncal arteries and embryonic liver from 21 to 58 days of development. The chronology of blood precursor cell emergence in these distinct tissues suggests a pivotal role in the settlement of liver hematopoiesis of endothelium-associated stem cell clusters, which emerge not only in the dorsal aorta but also in the vitelline artery. Anatomic features and in vitro functionality indicate that stem cells develop intrinsically to embryonic artery walls from a presumptive territory whose blood-forming potential exists from at least 24 days of gestation.

Key words: Hematopoietic stem cell, Hematopoiesis, Human, Yolk sac, Liver

INTRODUCTION

The development of the blood system in vertebrates is characterised by sequential switches in the sites where hematopoietic stem cells (HSC) undergo renewal, expansion and differentiation. An initial population of hematopoietic precursors, which originates in the extraembryonic mesoderm of the yolk sac (YS) or its equivalent, provides the developing embryo with a primitive generation of erythrocytes (Moore and Owen, 1967). It has long been assumed that yolk-sac-derived precursors were also responsible for the colonisation and, hence, hematopoietic development of the other blood-forming tissues that sequentially develop in the embryo and fetus, i.e. the liver, thymus, spleen, bursa of Fabricius of birds and, eventually, bone marrow (Moore and Metcalf, 1970; Andrew and Owen, 1978). Yet, experiments in amphibians and birds have demonstrated that hematopoietic precursors do not arise only in the YS during embryogenesis (Dieterlen-Lièvre and Le Douarin, 1993; Bechtold et al., 1992). In avian ontogeny HSC also originate in an intraembryonic region neighbouring the dorsal aorta, as clusters of cells that later yield all definitive erythrocytes and also give rise to multilineage progenitors (Dieterlen-Lièvre, 1975; Lassila et al., 1978). The homologous intraembryonic compartment in the *Xenopus* embryo is the dorsal lateral plate mesoderm which yields definitive adult erythroid and lymphoid populations (Maéno et al., 1985; Kau and Turpen, 1993). In this species, hematopoiesis derived from ventral mesoderm, a YS equivalent, is transitory and does not supply the adult with full hematopoietic potential.

The intraembryonic emergence of hematopoietic stem cells has also been described in the mouse paraaortic splanchnopleura (P-Sp), that is the presumptive aorta-gonad-mesonephros (AGM) region (Godin et al., 1993; Medvinsky et al., 1993). Histological analysis of mouse embryos aged 9.5 to 12 days showed hematopoietic cells clustered on the floor of the dorsal aorta and in the vitelline and umbilical arteries (Garcia-Porrero et al., 1995). In vitro culture of cells dissociated from the P-Sp or from the rest of the embryo body in the presence of hematopoiesis-supporting stromal cells demonstrated intraembryonic precursors to be exclusively located in the former tissue (Godin et al., 1995). Cells in the day-10 embryo AGM reconstitute the hematopoietic system in the long term when injected into irradiated mice (Muller et al., 1994). At 7.5 days, before circulation has connected the YS with the embryo, the P-Sp only contains stem cells with lymphoid potential (Cumano et al., 1996). Intraembryonic HSC emerge autonomously in situ, independently from the precursors emerging in the YS (Cumano et al., 1996; Medvinsky and Dzierzak, 1996).

We have previously identified in the preumbilical region of the 5-week human embryo a large population of hematogenous cells associated with the ventral endothelium of the dorsal aorta (Tavian et al., 1996). These cells exhibit a surface phenotype (CD45$^+$, CD34$^+$, Lin$^-$), in vitro behaviour (Tavian et al., 1996)

and gene expression pattern (Labastie et al., 1998) that are characteristic of primitive hematopoietic progenitors and constitute the largest local accumulation of such precursors ever described in the human hematopoietic system through development and adulthood (Tavian et al., 1995; Charbord et al., 1996). These properties, together with the spatiotemporal similarity that exists between the intraaortic hematopoietic cell clusters that arise in the human embryo and those described in animal models, led us to suggest that these cells are profoundly involved in the establishment of the human hematopoietic system. However, since blood already circulates when these endothelial aggregates appear, interchanges of precursors between embryo and YS may have already occurred, complicating the interpretation of the very origin of aorta-associated hematopoietic stem cells.

Using in parallel immunohistochemistry, in vitro culture and, in some instances, semithin section histology, the emergence of artery-associated HSC in the human embryo was therefore re-examined. A chronology of the hematopoietic processes occurring throughout the developing embryo in the context of alternate blood cell formation in the yolk sac and liver rudiment is here described for the first time in the human species. The results of this integrated study support the assumption that hematopoietic cells arise intrinsically in the ventral wall of human embryonic arteries.

MATERIALS AND METHODS

Human tissues

58 human embryos at 21 to 58 days of development were obtained immediately after voluntary terminations of pregnancy induced with the RU 486 antiprogestative compound. In all cases, informed consent to the use of the embryo in research was obtained from the patient, and the embryo was collected according to the guidelines and with the approval of both our national (CNE) and institutional (COPE) ethics committees. Gestational age was estimated on several developmental criteria: number of somite pairs at Carnegie stages 9-11, crown-rump size, limb-bud shape and eye pigmentation at stages 12-15 and 16-17, respectively (O'Rahilly and Müller, 1987) (Table 1). When needed, embryos where dissected in phosphate-buffered saline (PBS) under a microscope, using sterile microsurgery instruments.

Tissue processing for histology and immunostaining

Tissues were fixed for 1 hour at 4°C in PBS-4% paraformaldehyde (Sigma, St Louis, MO) (vol/vol), and rinsed in PBS for several hours and then twice in PBS-15% sucrose for at least 24 hours. Embryos were then embedded in PBS-15% sucrose, 7.5% gelatin and frozen at −70°C. 5-μm frozen sections were thawed and hydrated in PBS, and endogenous peroxidases were inhibited for 20 minutes in PBS containing 0.2% hydrogen peroxide. Sections were then washed with PBS-0.25% Triton X-100 (Sigma) and the primary antibody was added overnight at 4°C. After washing with PBS-Triton X-100, incubation was performed for 1 hour at room temperature, first, with biotinylated rabbit anti-mouse Ig antibody (DAKO, Glostrup, Denmark) and, subsequently, with peroxidase-labeled streptavidin (DAKO). Peroxidase activity was revealed 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 XAM neutral medium (BDH, Poole, UK). One 35-day embryo was processed for making semithin histological sections. Tissues were fixed for 2 hours in a 2.5% (w/v) glutaraldehyde solution buffered with 0.1 M sodium cacodylate (Sigma), then postfixed in 1% osmium tetroxyde (Sigma), dehydrated using increasing grades of

Table 1. Stages of the embryos analysed by immunohistochemistry

Carnegie stage	Number of somites	Developmental age (Days)	Number of specimens
9	1-3	20-21	1
10	4-12	22-23	8
11	13-20	24-25	4
12	21-29	26-27	15
13	30-35	28-30	5
14	*	31-32	5
15		33-36	4
16		37-40	3
17		41-43	1
Total			46

*From this stage on, the number of somites is difficult to determine and thus is not a useful criterion (from Moore and Persaud 1993).

ethanol, followed by propylene oxide and embedded in Araldite (Polysciences Inc, Warrington, PA). Semithin transverse sections (1 μm) were cut on an ultramicrotome and stained with 1% toluidine blue in 1% sodium borate buffer. All observations and photomicrographs were made on a Nikon Microphot-FXA microscope (Nikon, New York, NY).

Primary antibodies

Monoclonal antibodies to CD34 (HPCA-1) and CD45 (Hle-1) were purchased from Becton-Dickinson (San Jose, CA), and those to smooth muscle α-actin (SM α-actin, 1A4), CD68 (KP1) and glycophorin A (Gly-A, JC159) from DAKO. Fluorescein isothiocyanate (FITC)-labelled anti-CD34 (581) was obtained from Immunotech (Marseille, France).

Hematopoietic cell culture

Cells obtained directly from human embryos and yolk sacs and sorted CD34^{+} cells were cultivated on a pre-established monolayer of the Sys-1 mouse stromal cell line, previously shown to support long-term multilineage human hematopoiesis (Baum et al., 1992; DiGiusto et al., 1994; Young et al., 1996). Sys-1 stromal cells were plated in multiwell tissue culture plates 1 week earlier in medium [RPMI 1640 without phenol red (Gibco, Grand Island, NY), 10% fetal calf serum (FCS) (Hyclone, Logan, UT), 40 μM 2-mercaptoethanol (Sigma), penicillin (100U/ml) and streptomycin (100U/ml), 4 mM glutamine, 100 mM sodium pyruvate] and grown to confluence. Cocultures were initiated in the same medium supplemented with human hematopoietic cytokines [10 ng/ml recombinant human (rh) IL-3, 10 ng/ml rhIL-6, 25 ng/ml rh kit ligand (KL), and 10 ng/ml rh leukemia-inhibiting factor (LIF) (Pepro Tech Inc., Rocky Hill, NJ)]. Sorted CD34^{+} cells were seeded at 100-150 cells/well, in a 96-well plate, onto fresh Sys-1 layers in cytokine-supplemented medium. Half of the medium was replaced weekly. Plates were visually scored at weeks 4 through 6 for the presence of tightly clustered small hematopoietic cells (cobblestone areas). Wells with dispersed cells or only large cells were counted as negative.

In vitro explant culture

As described in Fig. 8 the yolk sac, liver and different portions of the aorta were seeded undissociated in wells of a 24-well plate containing Sys-1 stromal cells. In some cases, circulating blood was collected from the cardiac cavity with a pulled Pasteur pipette and assayed under the same culture conditions. Tissues were cultivated in toto for 24-48 hours, then mechanically dissociated through a 26-gauge needle and cultivated for 3-4 days in medium supplemented with cytokines.

Fluorescence-activated cell sorting

Cocultured hematopoietic cells were harvested from each well and

incubated for 20 minutes with FITC-anti-CD34 mAb on ice. Cells were analysed and sorted on a FACStar Plus cell sorter (Becton Dickinson) (Fig. 8).

RESULTS

We first described the emergence of the human hematopoietic system using immunohistological staining on YS and embryonic tissues ranging from 21 to 43 days of development. Expression of the CD34 antigen was used to trace developing endothelial and hematopoietic cells, the latter being discriminated by their coexpression of the CD45 pan-leukocyte surface molecule, and their immediate progeny identified with a panel of lineage markers. The histologic detection of progenitor cells was correlated with the hematopoietic potential in vitro of the tissues analysed.
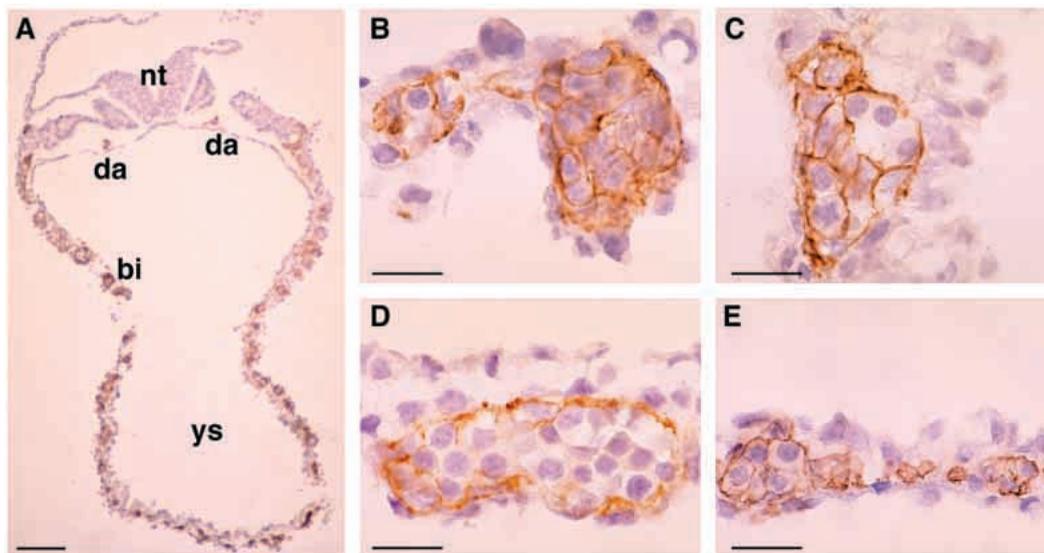
Blood islands in the yolk sac

In higher vertebrates, soon after the end of gastrulation, a network of "hemangioblastic" mesodermal cell aggregates that is at the origin of both vascular and hematopoietic systems develops in the extraembryonic area. In these originally solid clusters, the most peripheral cells flatten into endothelial cells, while most of the inner ones simultaneously disappear to open the first vessel lumens. Clumps of primitive mesodermal cells remain adherent to the newly formed vascular endothelium: these are stricto sensu the blood islands, where original hematopoiesis takes place (Sabin, 1920; Kessel and Fabian, 1985).

Incipient vasculogenesis and hematopoiesis in the human YS are difficult to study since they occur as early as day 18.5 of development (Bloom and Bartelmez, 1940). Indeed, in the earliest YS that we analysed (3 somites, 21 days), blood island

differentiation had already begun and three types of hemangioblastic mesodermal structures could be distinguished (Fig. 1A-D). The first and rarest one was still solid and characterised by a close physical association between emerging endothelial and hematopoietic cells, all or most of which were positive for CD34 surface expression (Fig. 1B). The second type consisted of a less compact, already hollow structure in which all flattened endothelial cells were CD34+, as were some rounded cells adhering to those (Fig. 1C). CD34-negative cells exhibiting erythroblast shape and expressing glycophorin A (Gly-A) (not shown) were also already present. The third type encountered was represented by differentiated capillaries bordered by CD34+ endothelial cells and containing erythroid cells, while CD34+ cells adhering to the endothelium were rare or absent (Fig. 1D,E). The yolk sac mesoderm further differentiates till day 24 (15 somites, stage 11), when neither hemangioblastic cell clusters nor intravascular blood islands are present anymore. At this stage, the YS is densely vascularized by vessels containing Gly-A-expressing erythroblasts and CD45+ cells (not shown). Since the heart starts beating around the 4-somite stage (deVries and Saunders, 1962), these cells are likely to be circulating.

### Onset of blood circulation and identity of early circulating cells

At 21 days (3 somites, stage 9), the embryo proper, analysed on serial transverse sections, was not found to contain cells expressing the pan-leukocyte CD45 antigen (not shown). However, cells resembling erythroblasts and expressing Gly-A were seen inside the cardiac cavity (Fig. 2A), suggesting that blood circulation is already established at this stage between the extraembryonic and intraembryonic compartments. Failure to detect the CD45 antigen on embryo sections is not technical, since at 21 days this protein is already evident on the surface

Fig. 1. Yolk sac blood islands. (A) Transverse section in a 3-somite human embryo (21 days) at the truncal level. In the embryo proper, the anti-CD34 antibody stains the endothelium in the paired dorsal aortae (da), ventrolateral to the neural tube (nt). In the extraembryonic yolk sac (ys), CD34 is expressed in hemangioblastic mesodermal tissues at various levels of differentiation, illustrated at a higher magnification (B-E). (B) A solid hemangioblastic cluster of cells expressing CD34. (C) A less compact structure with a developing lumen still contains clumps of CD34+ cells (blood islands) associated with the arising endothelium. A differentiated primary capillary bordered by CD34+ endothelial cells (D) contains circulating erythroid cells stained by Gly-A (E). Scale bars: (A) 200 μm, (B-E) 25 μm; bi, blood island.
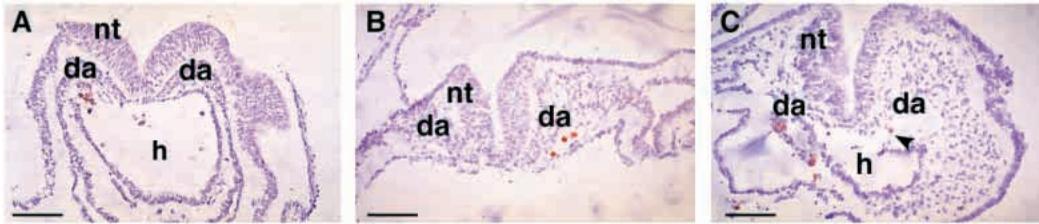
Fig. 2. First hematopoietic cells inside the embryo proper. (A) Cross section at the cardiac level through a 3-somite embryo (21 days). Some erythroid cells expressing glycophorin A are detected inside the heart (h). (B,C) Transverse sections through a 5-somite embryo stained for Gly-A and CD45, respectively. (B) Some erythroid cells are present inside the dorsal aorta (da) lumen in a postcardiac section. (C) Hematopoietic CD45+ cells inside the heart and dorsal aorta (arrowhead). Scale bar: 100 μm; nt, neural tube.
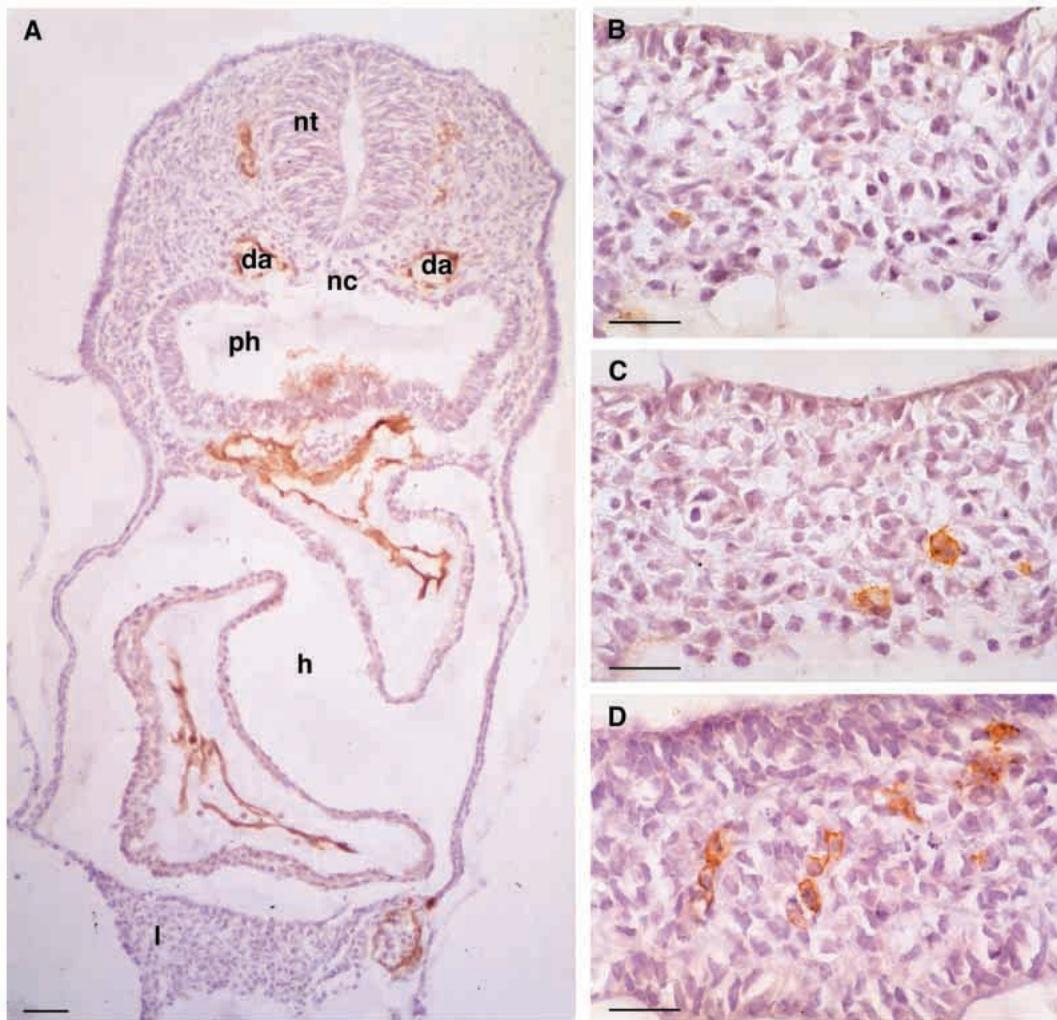
Fig. 3. Hematopoietic cells in the hepatic rudiment. (A) Cross section through a 12-somite embryo (23 days) at the thoracic level. Anti-CD34 stains the endothelium of the paired dorsal aortae (da), of capillaries around the neural tube (nt) and the endocardium lining the heart cavity (h). (B,C) Sequential sections through the liver in the same embryo stained for CD34 and CD45, respectively. No hematopoietic CD45+ cells (C) express CD34 (B). (D) Some Gly A+ erythroid cells are present in the hepatic rudiment. Scale bars: (A) 50 μm, (B,C,D) 25 μm; nc, notochord; ph, pharynx; l, liver.

of hematopoietic cells in yolk sac forming blood vessels (not shown). The first hematopoietic CD45+, CD34− cells were detected inside the embryo at day 22 of development (5 somites, stage 10), just following the onset of cardiac contractions. These cells were conspicuous inside the cardiac cavity (Fig. 2C) while, in the YS, some blood islands of type 1 or 2 were still present (see above). At this stage, erythroid cells (Gly-A+, CD34−, CD45−) are also detected in the lumen of the paired aortae (Fig. 2B). These observations confirmed that the onset of circulation between extraembryonic and intraembryonic compartments takes place at the 3- to 4-somite stage.

Hematopoietic cells in the embryonic liver

At the 10-somite stage (23 days, stage 10), the liver rudiment develops from an appendage of the duodenum that invades the septum transversum, as a hepatic plate, caudal to the heart. At this stage, no hematopoietic cells are yet detected by immunohistochemistry in the liver anlage (not shown). It is at the 12-somite stage (23 days, stage 10) that rare, scattered CD45-positive cells were first found in the hepatic rudiment (Fig. 3C). Staining of adjacent sections showed that these cells do not express the CD34 antigen (Fig. 3A,B); while, in other sections, CD34 is present on the surface of polygonal, elongated cells evoking endothelial precursors (not shown). At the same stage, Gly-A$^+$ erythroid cells were also seen in developing hepatic sinusoids (Fig. 3D).

By 26 days of development (24 somites, stage 12), dispersed CD45-positive cells can be detected around the neural tube, in the liver, in the thoracic and abdominal regions of the mesentery and in the mesenchyme surrounding the dorsal aorta (Fig. 4A). CD68$^+$ cells are distributed similarly in these territories, suggesting that most of these scattered hematopoietic cells belong to the monocyte/macrophage lineage (not shown). At the same stage, all circulating cells inside vessel lumens belong to the erythroid lineage, characterised by Gly-A expression (not shown). Round CD34$^+$ hematopoietic cells are first detected in the liver (2 or 3 per section) from 30 days (stage 13), often associated with the endothelium of capillaries which itself is dimly CD34-positive (Fig. 4F). Adjacent section staining shows that these cells coexpress CD45, and are therefore hematopoietic progenitors (Fig. 4E). From this stage on, hepatic CD34$^+$ cells increased progressively in number until the latest stage analysed, 17 (42 days), when the liver contains numerous scattered CD34$^+$ hematopoietic cells (12-15 cells on the largest sections) and constitutes the major hematopoietic organ inside the embryo (Fig. 4G and H).

Arterial clusters of hematopoietic progenitor cells

A brief description of the development of the human vascular system will help to localise arterial hematopoietic clusters inside the embryo. The two dorsal aortae appear at stage 9 of development (21 days, 1-3 somites) (Ingalls, 1920) and start fusing rostrally from stage 12 (25 days) in the trunk region. The fusion does not reach completion in the most caudal part of the embryo where the two paired vessels give rise to the left and right umbilical arteries. The vitelline artery appears at stage 13 (30 days) and connects the vitelline plexus (i.e. yolk sac blood vessels) with the blood vessels of the body proper (Evans, 1912).

Vitelline artery

From 30 to 36 days of development (stages 13-15), we detected several intraluminal hematopoietic cell clusters associated with the ventral endothelium of the vitelline artery. These aggregates are thicker in the region where the vitelline artery is connected with the aorta while, at more distal levels, they arise as tiny
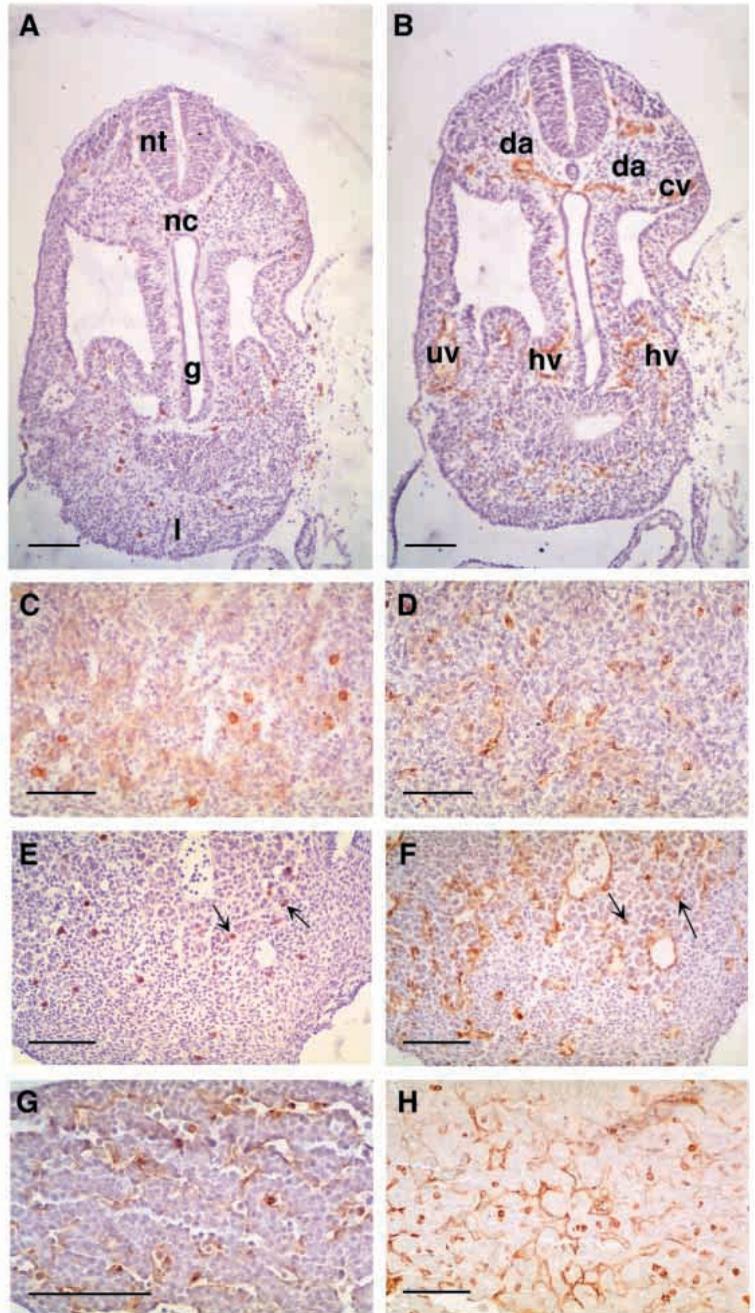

Fig. 4. Hematopoietic colonisation of the liver. (A,B) Transverse sections through a 26-day embryo stained for CD45 and CD34, respectively. CD45$^+$ hematopoietic cells are present in the liver, in vessels around the neural tube (nt) and in the paired dorsal aortae (da). The CD34 antibody stains the endothelium of all vessels: dorsal aortae, umbilical vein (uv), hepatocardiac veins (hv), cardinal veins (cv) and perineural capillaries. (C,D) Consecutive transverse sections through a liver at 27 days of development. No CD45$^+$ hematopoietic cells (C) express the CD34 antigen (D). The endothelium of capillaries and veins is weakly stained by the CD34 antibody (D). (E, F) Sequential transverse sections through a 30-day liver stained by CD45 and CD34 antibodies, respectively. The CD34 antigen is present on the surface of all flattened endothelial cells. Rare rounded hematopoietic CD45$^+$ cells also express the CD34 antigen (arrows). (G,H) Transverse sections through the liver at 32 and 42 days of development, respectively, stained by anti-CD34 show several round hematopoietic cells inside the vessels and in the hepatic parenchyma. Scale bar: 100 μm; g, gut; nc, notochord.

groups of cells (Fig. 5D and F). As for aortic aggregates (Tavian et al., 1996), all the cells inside these clusters coexpressed the CD45 and CD34 antigens at all stages studied (13 to 15) (Fig. 5G,H).

Dorsal aorta

The hematopoietic clusters in the dorsal aorta are composed of round cells expressing CD45 and CD34 (Fig. 5G and H) (Tavian et al., 1996). However, these clusters, which are also always associated with the ventral endothelium, emerge earlier (stage 12) and persist longer (stage 16) than the vitelline artery aggregates (Fig. 10). Aorta-associated progenitors appear at 27 days of development as scattered groups of a few cells at the truncal level of the fused aorta and are precisely located in the preumbilical region (Fig. 5B). Groups of 2-3 cells are often also detected in a more rostral region, where the aorta is still bifurcated (Fig. 5A). Beginning at day 30 of development (stage 13), the clusters encountered at the bifurcation of the vitelline artery increase in size (Fig. 5C,D) to reach several hundreds of cells at stages 14 and 15 (Fig. 5E,F). At those stages, other clusters also appear more caudally (Fig. 5G). At subsequent stages of development, aortic clusters undergo gradual decrease till day 40 (stage 16), the latest at which they could be detected on a total of 20 embryos examined (not shown).

Morphological analysis on semithin transverse sections of a 35-day human embryo (stage 15) revealed specific features of the endothelium in the ventral portion of the aorta bearing the hematopoietic clusters. Endothelial cells underlying the aggregated hematopoietic cells at both ends of aortic clusters constitute a regular lining similar to that encountered in the dorsal wall of the vessel (Fig. 6A,D). In contrast, the endothelial barrier appears to be interrupted in the central part of the cluster where hematopoietic cells seem to be directly connected with subaortic mesoderm. These observations suggest the sprouting of hematopoietic cells into the vessel lumen, from the underlying tissue, through disrupted endothelial wall and/or from dedifferentiated ventral endothelial cells (Fig. 6B,C).
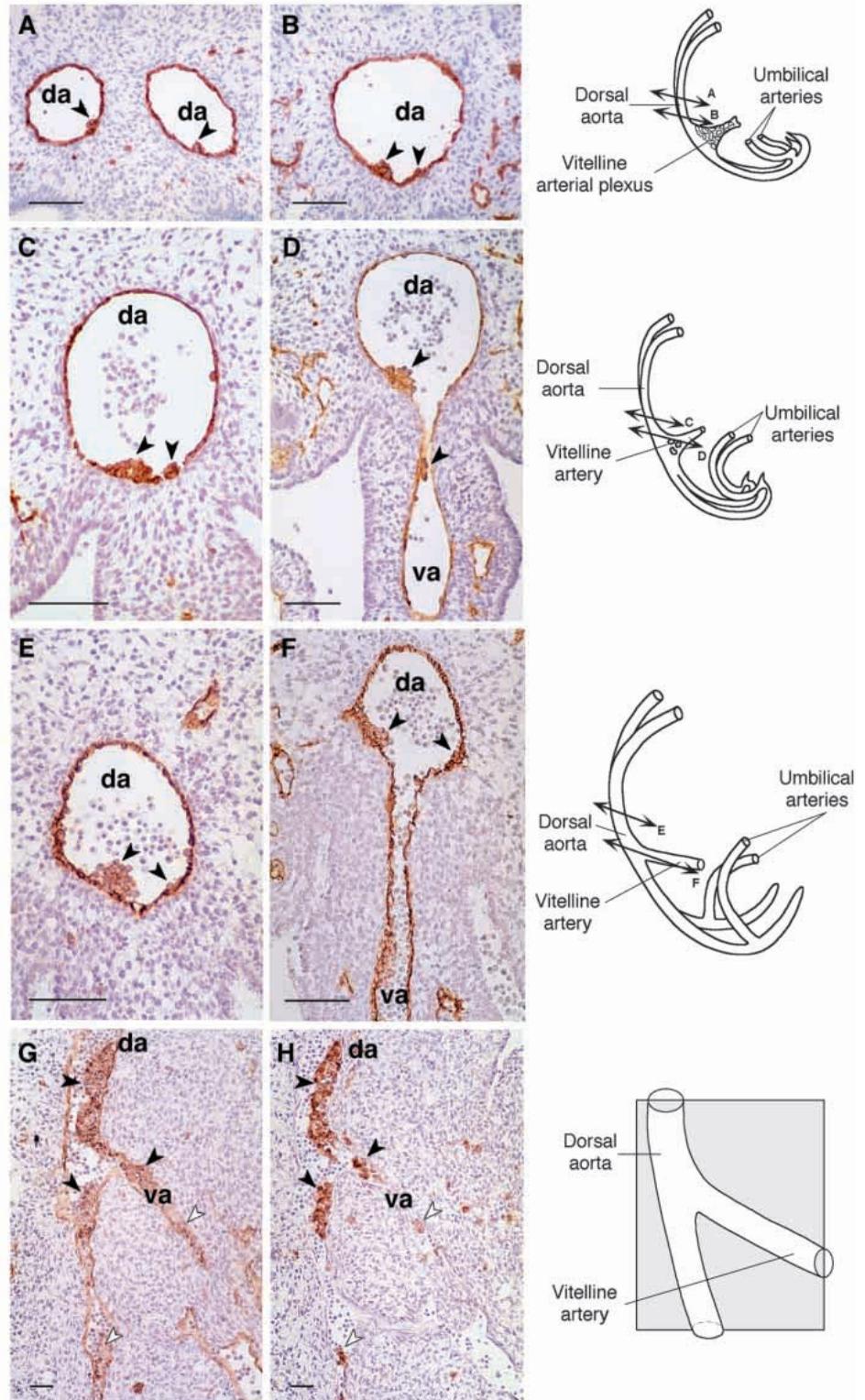
Fig. 5. Hematopoietic cell clusters associated with the arterial endothelium. Transverse sections (positions shown on diagrams) through the dorsal aorta (da) of a 27- (A,B), 32- (C,D) and 36-day (E,F) embryos immunostained by anti-CD34. Black arrowheads show the hematopoietic CD34+ cell clusters associated with the endothelium on the ventral sides of the dorsal aorta and vitelline artery (va). (G,H) Sagittal sections through a 36-day embryo stained for CD34 and CD45, respectively. The section plane is represented by a shaded rectangle on the sketch to the right of H. All hematopoietic clusters (arrows) are CD34+ (G) and CD45+ (H). White arrowheads indicate hematopoietic aggregates in a more distal part of the vitelline artery and a more caudal part of the dorsal aorta. Scale bar: 100 μm.
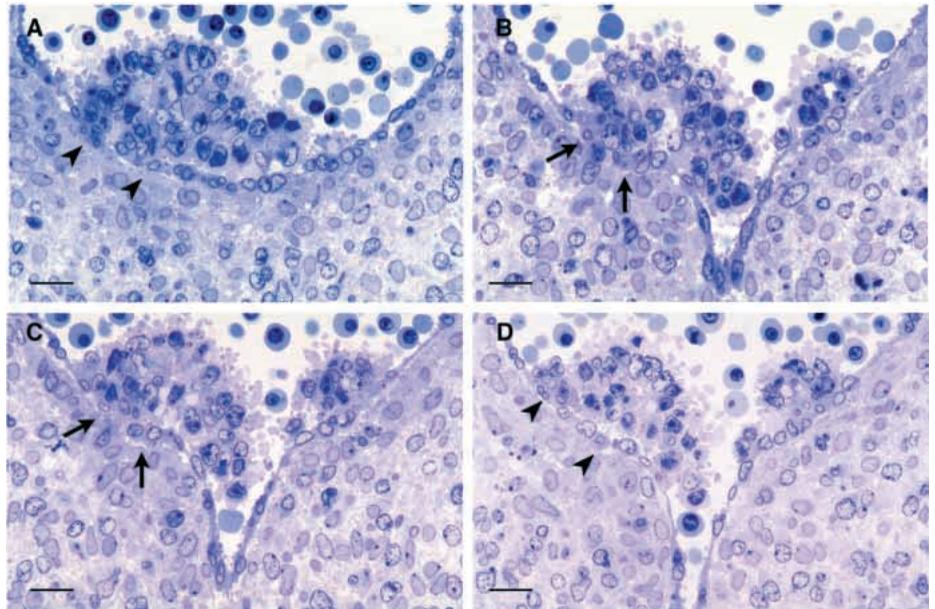
Fig. 6. High magnification of semithin transverse sections through the aorta of a 35-day embryo at the truncal level. (A,D) Anterior and posterior ends of aortic clusters showing intact endothelial lining (arrowheads) underneath hematopoietic cells. (B,C) In the central part of the cluster the endothelial barrier seems to be interrupted, and hematopoietic cells directly connected with the subaortic mesoderm (arrows). Scale bar: 20 μm.

Phenotype of late circulating cells

By stage 13 (day 30 of development), after the emergence of arterial hematopoietic clusters, and until day 43, blood still carries mostly cells of the erythroid lineage. Some rare CD45^{+} cells are also detected in the lumen of large vessels and in the cardiac cavity, while CD34^{+} circulating cells are even less frequently observed (in the range of 1-5 cells per embryo). Free clumps of a few CD34^{+} CD45^{+} cells were occasionally encountered inside the aorta, close to the region bearing endothelium-adherent hematopoietic cell clusters (not shown).
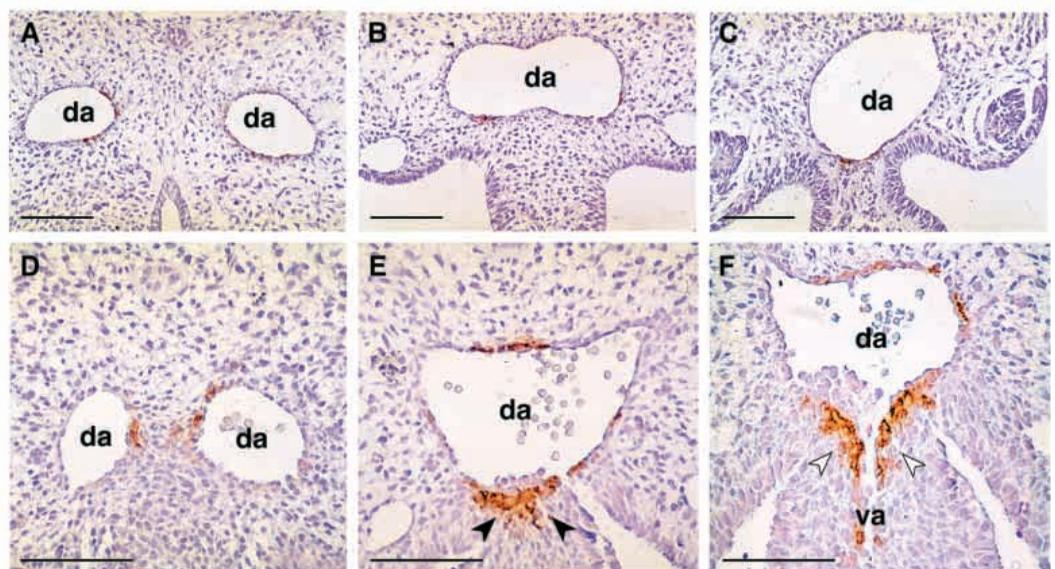
SMαA expression

We have analysed the expression in the hematopoietic territories described above of smooth muscle α-actin (SMαA), defined as a marker of hematopoiesis-supporting bone marrow stromal cells in the human species (Galmiche et al., 1993).

Stage 12 (days 26-27)

Only a few smooth muscle cells are detected in the embryo. A faint and discontinuous SMαA staining is present in the mesenchyme underlying the endothelium of the descending aorta. SMαA^{+} cells are located between the paired aortae in the cervical and thoracic regions (Fig. 7A), whereas they are restricted to the ventral side of the vessel in the abdominal segment of the fused aorta (Fig. 7B and C).

Stage 13 (days 28-30)

At this stage, the endothelial tube in most of the dorsal aorta is surrounded by a discontinuous monolayer of SMαA^{+} cells. At the cervical and thoracic levels, the ventromedial side of each aorta is more intensely stained than the dorsolateral one. Slightly caudally to the point where the paired aortae fuse into a single vessel (at the thoracic level), the ventral side only is associated with two layers of SMαA-positive cells, whereas the

Fig. 7. Expression of smooth muscle α-actin (SMαA) in the hematopoietic arteries. Transverse sections through a 27- (A-C) and a 33-day (D-F) embryo stained by SMαA. Arrowheads show the multilayered condensation of cells expressing SMαA in the segments of the dorsal aorta (da) and vitelline artery (va) bearing hematopoietic clusters. Scale bar: 50 μm.

other parts are lined with a monolayer of SMαA^{+} cells (not shown). It is noteworthy that such a thicker ventral condensation of cells expressing smooth-muscle actin is strictly restricted to vessel segments bearing hematopoietic clusters, be they in the aorta or in the vitelline artery.

Stages 14 and 15 (days 30-46)

SMαA exhibits the same expression pattern as at stage 13. SMαA^{+} cells are much more numerous and arranged in 2-3 layers in the vicinity of endothelial cells associated with hematopoietic cell aggregates (Fig. 7E,F). A few SMαA[+] are also present on the lateral inner sides of the paired aortae in the cervical regions (Fig. 7D). In contrast, SMαA^{+} cells underlying the non-hematopoietic ventral endothelium, or the whole dorsal one in both the aorta and vitelline artery, were always single-layered, as in other arteries of the embryo body (not shown).

In vitro hematopoietic activity of the human yolk sac and intraembryonic tissues

We used an in vitro coculture system to draw a functional correlate to the above-described distribution of hematopoietic progenitors in the human embryo. Tissues were dissected from embryos and directly seeded in toto on a confluent monolayer of Sys-1 cells, which support hematopoiesis from human pre- and postnatal stem and progenitor cells (Baum et al., 1992; DiGiusto et al., 1994; Young et al., 1996). Undissociated explants attached to the surface of stromal cells and spread into layers of fibroblast-like cells. Hematopoietic cells were detected morphologically but only transiently in such cultures, where other unrelated adherent cells rapidly overgrew and inhibited developing blood cells (not shown). To circumvent this problem, dissected explants were allowed to attach to Sys-1 stromal cells for 24 hours and were then dissociated mechanically in situ by pipetting (Fig. 8). Resulting cell aggregates were left in vitro for 3-4 additional days, to allow new adherent layers to form, of which those showing morphological evidence of hematopoiesis were further processed. CD34^{+} cells were sorted by flow cytometry from these cultures and seeded, at 100-150 cells/well, in a 96-well plate, onto fresh Sys-1 cell layers.

The rostral, truncal and caudal segments of the aorta, as well as liver, YS and circulating blood were individually cultivated under the conditions described above. Cells were cultured for 30-40 days and the hematopoietic potential of each territory was assayed through the ability of CD34^{+} cells sorted therefrom to establish a long-term culture in vitro. Culture wells were evaluated visually

Table 2. Hematopoietic potential of human embryonic territories

| Embryo age (days)	Aorta*			Liver	Yolk sac	Heart blood
	Anterior	Median	Posterior			
24	0/1	0/1	1/1‡	0/1	1/1	ND
27	0/1	1/1	0/1	0/1	1/1	0/1
30-32	0/3	3/3	0/2	0/2	3/3	0/2
33	0/1	1/1	0/1	1/1	1/1	0/1
42-58§	ND	ND	ND	4/4	ND	ND

CD34^{+} cells sorted from the human yolk sac and intraembryonic tissues, precultivated in toto were cocultured with Sys-1 murine stromal cells for 30-40 days. Frequencies of cultures containing hematopoietic cell colonies, per total number of tissues tested at various stages, are indicated.

ND, not determined.

*Anterior, median and posterior regions correspond, respectively, to the rostral tract before the fusion of the artery, the preumbilical and the postumbilical segments.

‡At this stage, the posterior region includes the portion of the aorta that will give rise to the umbilical region.

§At these stages, the liver only was dissected and dissociated cells were used directly for cell sorting.
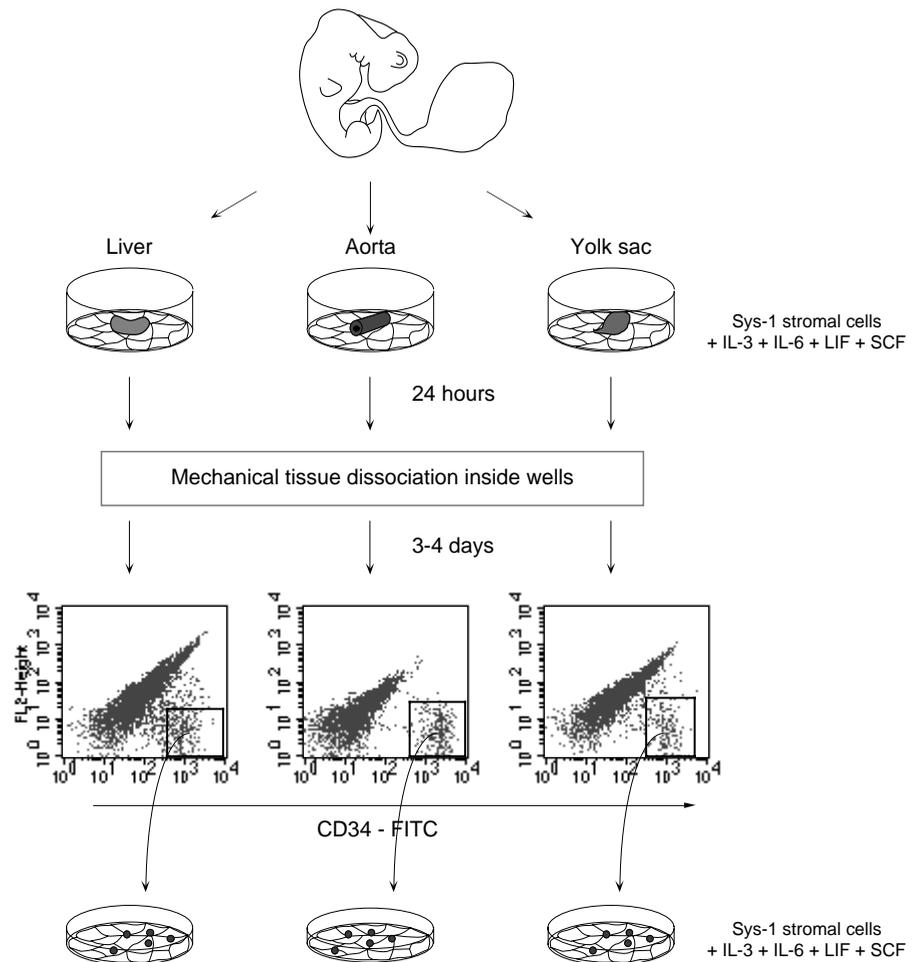
Fig. 8. Scheme of in vitro assays for hematopoietic progenitors in human embryonic territories. Dissected explants from 24- to 58-day human embryos were cultured for 24 hours on a monolayer of Sys-1 stromal cells, then mechanically dissociated and left in vitro for 3-4 additional days. CD34^{+} cells were sorted from the latter cultures and seeded onto fresh Sys-1 cells for 30-40 days.

and considered positive when containing tightly packed clusters of hematopoietic cells (cobblestone areas). Table 2 shows the results obtained from 11 embryos ranging from 24 to 58 days of development. Hematopoietic precursors were detected in the liver exclusively after the 32nd day of development. By contrast, the aorta dissected from the preumbilical region yielded hematopoietic colonies at all stages analysed, from 27 to 33 days of development, while the other parts of the aorta never exhibited any hematopoietic potential. Hematopoietic colonies were generated by yolk sac explants at all stages analysed, while cardiac blood never gave rise to hematopoietic growth in vitro. The earliest embryo studied was 24 days old (17 somites), from which the liver and YS were removed before cutting the remainder of the embryo
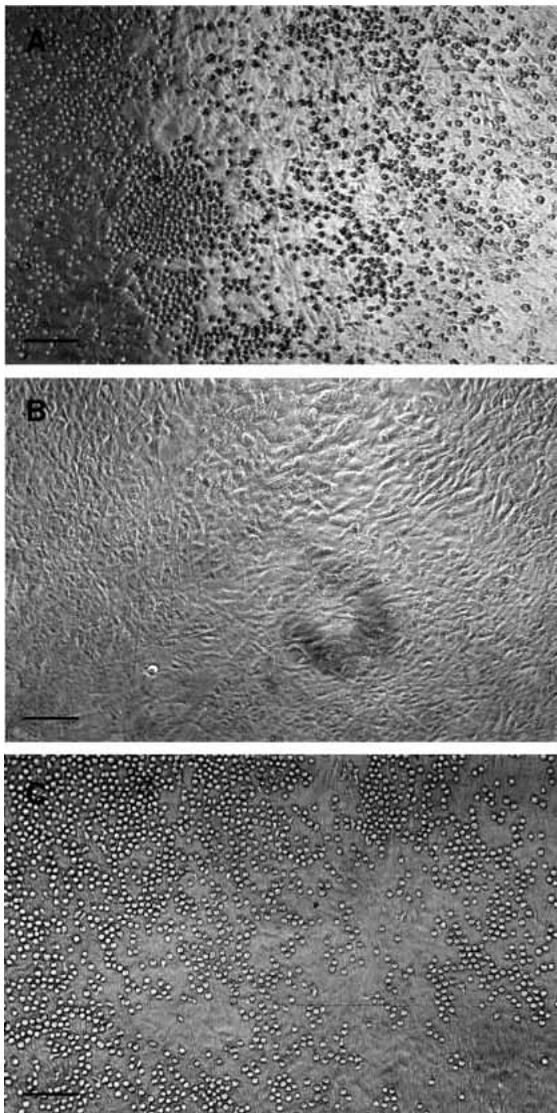

Fig. 9. Coculture of hematopoietic cells with Sys-1 stromal cells. CD34+ cells sorted from tissues of a 24-day human embryo were cultured for 30 days on a Sys-1 cell layer. No hematopoietic cells developed from the anterior (B) and median (not shown) segments of the embryo. The most posterior region of the embryo (C) and the yolk sac (A) gave rise to hematopoietic cell colonies adhering to the stromal cells. Scale bars: 100 μm.

body transversely into three. All tissues were cultivated intact in separate wells under the conditions described above for 4 days, then dissociated and the cells seeded into three fresh wells. Fig. 9 shows that hematopoietic cell growth was restricted to wells seeded with cells from the YS and from the most posterior part of the embryo, which contains the presumptive hemogenic portion of the aorta.

DISCUSSION

We have suggested in a previous article that hematopoietic precursors emerge inside the human embryo proper (Tavian et al., 1996). Our assumption was based on the detection of several hundreds hematopoietic CD34+ cells associated with a short segment of the ventral aspect of the dorsal aorta at 5 weeks of gestation. Furthermore, the culture of these dissected hematopoietic aortic clusters onto a stromal cell line supporting hematopoiesis generated much higher number of CD34+ cells and progenitors than any other part of the embryo, liver included. These findings coincided strikingly with the demonstration that the aortic region is a primary hematopoietic territory in bird and mouse embryos (Dieterlen-Lièvre and Le Douarin 1993; Dieterlen-Lièvre, 1997).

In the present report, we provide arguments suggesting that hematopoiesis in the human liver, which is the major site of HSC differentiation during vertebrate prenatal development, is dependent on colonisation by progenitors originating in this region of the dorsal aorta. Analysis of immunostained sections shows that, from 24 days, the YS does not contain hemangioblastic cell clusters or intravascular blood islands expressing CD34. In contrast, the colonisation of the liver by CD34− erythromyeloid progenitors begins on the 23rd day of development (see Fig. 3). Since the onset of circulation between the extraembryonic and intraembryonic compartments takes place at 21-22 days, hematopoietic cells detected inside the hepatic rudiment at these early stages may derive from the YS. This is in good agreement with the hypothesis formulated by others proposing the migration of hematopoietic progenitors from a generation site (YS) to a colonisation site (liver) via circulating blood (Migliaccio et al., 1986). However, the concurrent absence of hematopoietic CD34+ cells in the bloodstream, both in the embryo and in the YS, may indicate that circulating cells do not contain HSC and that the liver is colonised by hematopoietic stem cells of other origins. We show here that the first hematopoietic CD34+ cells appear in the liver at 30 days of development, closely following the emergence of hematopoietic CD34+ cells in the neighbouring ventral wall of the dorsal aorta, which occurs at day 27. The number of precursors detected in the hepatic rudiment increases concurrently with the expansion of these arterial cell clusters (Fig. 10), suggesting a contribution of the latter to the establishment of hepatic hematopoiesis. Cell culture assays confirm that the liver contains cells able to establish hematopoiesis in vitro only after day 32 (Table 2), when intraembryonic clusters reach maximal size. The caudal portion of the embryo, which includes the presumptive hemogenic aorta, possesses hematopoietic activity as early as day 24. Blood circulation has then just started and it is unlikely that this early activity arises from cells emigrated from the YS via the bloodstream. Moreover, this potential is absent from all
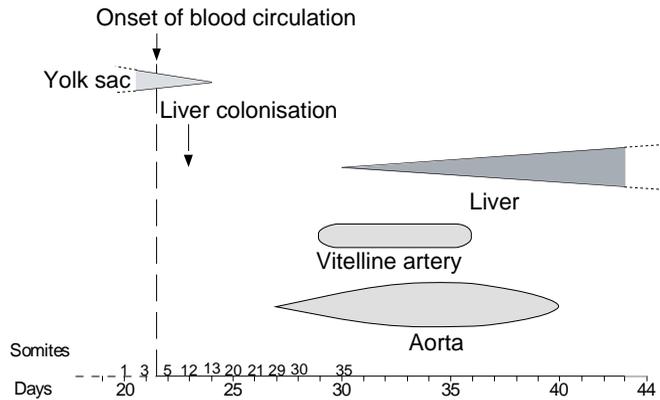
Fig. 10. Chronology of appearance of hematopoietic CD34$^+$ precursors in the developing human embryo.

other embryonic tissues analysed at the same stage, including other portions of the aorta and the liver, strengthening the notion that these immature hematopoietic progenitors emerge in situ. Yet, hematopoietic colonies developed in vitro from YS explanted even at later stages, when no CD34$^+$ cells were detected by immunohistochemistry in that extraembryonic appendage. While this activity may reflect the presence of circulating progenitor cells of intraembryonic origin, we have also shown that intravascular CD34$^+$ cells are only rarely found and that blood never possesses hematopoietic activity in vitro (Table 2). The restriction to the YS of such blood-borne progenitors could be due to the presence in that tissue of an intricate network of vessels and capillaries enhancing their mechanical retention. The presence of hematopoietic activity in the animal yolk sac at stages when circulation connects extraembryonic and intraembryonic compartments is still debated (Auerbach et al., 1996; Palacios and Imhof, 1996; Yoder et al., 1997). Cumano et al. (1996) have recently shown that the splanchnopleura, dissected from the mouse embryo before the onset of circulation, contributes to the myeloid and lymphoid lineages. By contrast, YS from the same embryos were unable to generate lymphoid progeny, suggesting that, as in birds, mouse intraembryonic progenitors are the founders of definitive hematopoiesis.

While the presence of endogenous pluripotent progenitors in embryonic non-hepatic territories confirms earlier observations (Huyhn et al., 1995; Tavian et al., 1996), we also define here, for the first time, the precise spatial localisation and chronology of appearance of these stem cell populations (Fig. 10). Our data, based on the observation of a large number of embryos, confirm that endothelium-associated hematopoietic cell clusters emerge inside a well-defined segment of the dorsal aorta, but also develop in the branching vitelline artery. This was already observed in the mouse (Garcia-Porrero et al., 1995) and underlines another striking similarity between the arterial clusters detected inside human and animal embryos. Moreover, we observed that smooth muscle α-actin exhibits peculiar expression underneath arteries harbouring hematopoietic cell clusters. In that region, several layers of SMαA$^+$ cells are condensed. Previous studies in vitro (Galmiche et al., 1993) had shown that SMαA is expressed in human bone marrow stromal cells that support hematopoiesis. The herein described accumulation of SMαA-

expressing mesenchymal cells around arterial cell clusters is therefore provocative. It has been also recently shown that HCA, a homologue of ALCAM (activated leukocyte cell adhesion molecule) normally present at the surface of the most primitive human hematopoietic CD34$^+$ cells (Uchida et al., 1997), is also expressed by stromal cells in prenatal and postnatal blood-forming tissues, including mesenchymal cells underneath aortic hematopoietic clusters (Cortés et al., 1999). On the contrary, observation of semithin sections through the dorsal aorta has shown that the ventral endothelium undergoes morphological changes and even disappears in the hematopoietic region of the vessel. This suggests the passage of hematopoietic cells from the subvascular mesodermal region toward the lumen of the aorta and/or the transdifferentiation of preexisting endothelial cells. Altogether these results point to a possible function of endothelial and mesenchymal cells located underneath the arterial clusters in hematopoietic stem cell emergence and expansion. In the mouse, AGM-derived CD34$^+$ endothelial cell lines mediate self-renewal of HSC, retaining the ability of these cells to competitively repopulate the bone marrow of lethally irradiated animals (Ohneda et al., 1998). Others have shown that endothelial VE-cadherin$^+$ cells sorted from embryos and YS exhibit lymphohematopoietic potential in vitro, suggesting the endothelial origin of artery-associated hematopoietic cell clusters (Nishikawa et al., 1998).

In conclusion, we propose the following scheme for human early hematopoietic ontogeny. After the onset of circulation, YS hematopoietic progenitors committed to the myeloerythroid pathway migrate to the liver at 24-25 days. Only after the emergence of intraembryonic vascular cell clusters (27 days) is the liver also colonised by CD34$^+$ precursors, which give rise to definitive hematopoiesis. Although the real contribution of HSC from embryonic and yolk sac origins in the establishment of definitive hematopoiesis is still to be definitely assessed, the results presented here, as well as extrapolated animal studies, highlight the crucial role of hematopoietic intraembryonic clusters in the ontogeny of the human hematopoietic system.

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