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Original Article

Multilineage hematopoietic progenitor activity generated autonomously in the mouse yolk sac: analysis using angiogenesis-defective embryos

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ABSTRACT The capacity of the yolk sac to generate multilineage, adult-type hematopoiesis was investigated *in vivo* using vascular endothelial-cadherin deficient embryos. In these mutants, the yolk sac is not connected to the vasculature of the embryo and therefore all hematopoietic activity detected therein is intrinsic to the yolk sac and not derived from intraembryonic sources. At embryonic days 9.5 and 10.5, the yolk sac contains blood cells from the first wave of hematopoiesis, i.e. primitive erythrocytes and monocytes, but also multipotent progenitors from definitive hematopoiesis and a few granulocytes. Reverse transcription-polymerase chain reaction analysis revealed expression of specific genes of all lineages except lymphoid cells. Moreover, hematopoietic colony assays showed the existence of committed progenitors of the second wave of embryonic hematopoiesis, namely for definitive erythrocytes, megakaryocytes, granulocytes and monocytes. Conversely, the number of lymphocytes after lymphoid culture was insignificant. Our data provide evidence for multilineage hematopoiesis (but not lymphopoiesis) in the yolk sac in the absence of seeding from the embryo. The small number of definitive mature blood cells indicates however that the yolk sac is not an effective environment for the terminal differentiation of committed progenitors from the second wave of hematopoiesis.

KEY WORDS: *hematopoiesis, yolk sac, progenitor, transgenic mice, vascular endothelial-cadherin*

Introduction

During mouse development, the first site of active hematopoiesis is the yolk sac (YS), where red blood cells can be detected as early as embryonic day (E)7.5 (Moore and Metcalf, 1970). This primary hematopoiesis appears in mesoderm-derived blood islands in the YS wall and produces primitive nucleated erythrocytes and macrophages. Once the vitello-embryonic circulation is established at E8.5, these cells are delivered into the circulatory system of the embryo until they are replaced at E10.5 by a second wave of blood cells resulting from adult-type hematopoiesis (for review, see Morales-Alcelay *et al.*, 1998; Cumano and Godin, 2001; Orkin and Zon, 2002). At this time of development, hematopoiesis is established in the fetal liver and generates multiple blood cell types, i.e., enucleated erythrocytes synthesizing adult globins, monocytes, granulocytes, megakaryocytes and lymphoblasts. The liver is the principal organ supporting blood cell production until hematopoiesis initiates in the bone marrow at the end of gestation. It is well established that the liver is not a site of emergence of hematopoietic progenitors and depends on colonization by circulating cells. However, the origin of adult hematopoietic progenitors has been a question of debate for the last thirty years.

The kinetic data of Moore and Metcalf (Moore and Metcalf, 1970) led to the hypothesis that committed adult-type progenitors found in the liver originated initially in the YS. Furthermore, the existence of definitive erythroid progenitors in the YS prior to vascular communication supported this view (Wong *et al.*, 1986). Later on, two groups identified an intraembryonic site of emergence of definitive hematopoietic progenitors in the paraaortic

Abbreviations used in this paper: AGM, aorta-gonad-mesonephros; βH1, embryonic β-globin; β-maj, adult β-globin; BFU, burst-forming unit; CFU, colony-forming unit; E, embryonic day; FCS, fetal calf serum; GEMM, granulo-erythro-myelo-megakaryocytic; HPRT, Hypoxanthine phosphorybosyltransferase; IL, interleukin; mAb, monoclonal antibody; MGG, May-Grünwald-Giemsa; Mk, megakaryocyte; MPO, myeloperoxydase; PBS, phosphate buffer saline; PECAM, platelet/endothelial cell adhesion molecule; PF4, platelet factor 4; P-Sp, paraaortic splanchnopleura; SCF, stem cell factor; VE-cadherin, vascular endothelial-cadherin; YS, yolk sac.

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splanchnopleura (P-Sp)/aorta-gonad-mesonephros (AGM) region (Godin *et al.*, 1993; Medvinsky *et al.*, 1993). Organotypic culture as well as *in vivo* grafting experiments into lethally-irradiated mice revealed that the P-Sp/AGM but not the YS contained a pluripotent hematopoietic potential (Medvinsky and Dzierzak, 1996; Cumano *et al.*, 2001). The P-Sp/AGM region was shown to develop lymphohematopoietic colonies *in vitro*, when removed before the circulation was established, whereas the YS only developed a lymphoid potential following communication with the embryonic vasculature (Cumano *et al.*, 1996). These data suggested that multipotent stem cells colonize the YS from intraembryonic sites, thereby restricting the definitive hematopoietic potential to the P-Sp/AGM. In addition, colony-forming unit (CFU)-spleen progenitor activity was observed in the E9.0 AGM, and long-term repopulating hematopoietic stem cells in adult recipients were detected at E10.0 in the same location. However, this activity could only be detected in the YS at E11.0 (Medvinsky *et al.*, 1993; Muller *et al.*, 1994). These findings tended to show that the emergence of hematopoietic progenitors occurs in two different sites, the YS being restricted to primitive erythromyelopoiesis and the P-Sp/AGM responsible for adult multilineage hematopoiesis.

This hypothesis was challenged by several groups. Yoder and collaborators reported that isolated cells from E9.0 YS repopulated erythroid, lymphoid and myeloid lineages long-term upon transplantation into newborn recipient animals (Yoder and Hiatt, 1997; Yoder *et al.*, 1997a; Yoder *et al.*, 1997b). Furthermore, adult repopulating hematopoietic stem cells could be obtained after 4 days of coculture of E8.5 YS cells with an AGM-derived stromal cell line (Matsuoka *et al.*, 2001). In another report, endothelial cells from the E9.5 YS were shown to generate all blood cell types, including lymphocytes, suggesting that the YS endothelium might be a source of multipotent hematopoietic stem cells (Nishikawa *et al.*, 1998). Whether the potential of endothelial cells to generate hematopoietic progenitors is exercised *in vivo* remains unclear (Ogawa *et al.*, 2001). Finally, kinetic analyses of precisely timed embryos revealed that adult-type erythromyeloid progenitors as well as high proliferative hematopoietic precursors are located in the YS before they can be detected in the blood or embryo proper (Palis *et al.*, 1999; Palis *et al.*, 2001). These findings further support the prevalent role of the YS in the generation of multipotent hematopoietic stem cells.

In fact, the major difficulty with which these studies were faced, and which may explain these controversies, is the establishment of the vitello-embryonic circulation as early as E8.5, before the appearance of substantial numbers of committed progenitors (i.e., CFU) in either compartment. From this age, cells circulating in the vascular system may comprise hematopoietic or hemangioblastic stem cells or even hemogenic endothelial cells. Although elegant and sophisticated studies have been performed to circumvent this issue, direct evidence of multipotent hematopoietic stem cell activity intrinsic to the YS at later stages of development is still lacking. In this paper, we took advantage of angiogenesis-defective embryos, obtained after vascular endothelial (VE)-cadherin gene inactivation (Gory-Faure *et al.*, 1999), to explore the hematological situation of the insulated YS after E8.5. In homozygous mutant animals, YS blood islands do not assemble into a primary plexus and there is no vascular communication between the YS and the embryo proper. Although the embryo rapidly degenerates after E9.5, the YS in the vicinity of the maternal decidua stays alive until the embryo is resorbed at E12.0. In this context, we could detect, in addition to primitive erythrocytes and monocytes, megakaryoblasts and a few granulocytes. Moreover, by using various progenitor assays, we could demonstrate the existence of CFU activity for definitive erythrocytes, megakaryocytes, granulocytes and monocytes in the YS. These data were confirmed by RT-PCR amplification of lineage-specific mRNAs. Our results definitely prove that the YS is a source of multipotent progenitors. They also suggest that the YS environment is not appropriate for complete hematopoietic differentiation except for primitive blood cells.

Results

Hematopoietic Cell Content of the YS

The absence of a vascular communication between the YS and the embryo proper of VE-cadherin deficient mice was established by different observations: (i) lack of visible blood cells within the intraembryonic vasculature, (ii) no continuous vessel in the vitelloembryonic stalk, (iii) absence of PCR amplified products for *embryonic β-globin (βH1)* cDNA within the embryo proper (Gory-Faure *et al.*, 1999). Somite number in the mutant embryo never exceeded 20 and we could never observe a fusion of dorsal aortae. These features suggest that embryonic development is arrested before any intraembryonic hematopoietic progenitors are generated. However, VE-cadherin deficiency did not impair primitive hematopoiesis in the YS. Primitive erythrocytes and macrophages represent approximately 50% and 10% of total cells respectively, from collagenase-treated YS cells. These percentages were iden-

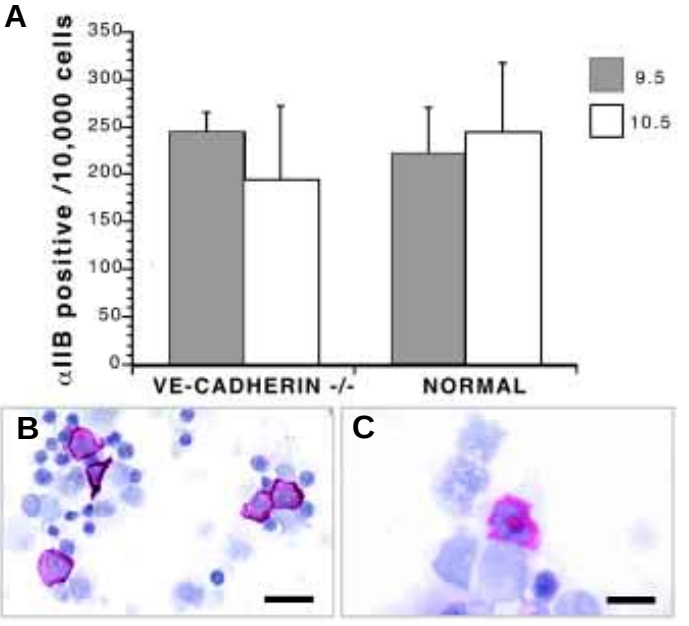

Fig. 1. αIIb and Gr1 immunolabelling of *VE-cadherin*$^{-/-}$ YS. *YS were harvested at E9.5 (A-C) or E10.5 (A) and cells were dissociated by collagenase treatment and cytocentrifuged. Megakaryoblasts and multilineage progenitors were immunolabelled with anti-αIIb mAb* **(A,B)** *and granulocytes with Gr-1 mAb* **(C)**. *In (A), data are the mean (+ SEM) of three independent YS counts. The frequency of αIIb^{+} cells was similar in normal and mutant YS. Few granulocytes could be detected in either YS types. Bars: 20 μm in (B); 10 μm in (C).*

Embryonic day		9.5		10.5		11.5		Bone Marrow
Lineages	Genes	+/+	-/-	+/+	-/-	+/+	-/-	
Multipotent	αIIB							
Megakaryocytic	PF4							
Granulocytic	MPO					ND	ND	
Erythroid	βH1							
	βmaj							
Lymphoid	RAG 2							
Controls	VE-cadherin							
	HPRT							

Fig. 2. RT-PCR analysis of lineage-specific gene expression in YS. *RNA from YS of the indicated embryonic ages were reverse transcribed and subjected to PCR. Semi-quantitative amplification conditions were determined for each primer set. Shown are Southern blots of RT-PCR products probed with the corresponding internal probe. The figure shows representative results from one of three experiments performed with independent YS pools. Bone marrow RNA were used as positive controls. N.D.: not determined.*

tical at both E9.5 and E10.5 and in normal or *VE-cadherin*$^{-/-}$ YS. YS cells were immunolabelled with anti-αIIb, a marker originally described as megakaryocyte-specific but recently identified on multipotent hematopoietic progenitors as well (Corbel and Salaün, 2002; Mikkola *et al.*, 2003). Numerous αIIb^{+} cells were observed among *VE-cadherin*$^{-/-}$ YS cells. Furthermore, a similar proportion of αIIb^{+} cells was detected (~2.5% of total cell number) in *VE-cadherin*$^{-/-}$ or normal YS, at E9.5 or E10.5 (Fig. 1A), suggesting that all αIIb^{+} cells of the normal YS are produced locally. These cells did not harbour the morphological features of mature megakaryocytes, which normally have a large cytoplasm and a multilobed nucleus (Fig. 1B) and only some were also positive for the late megakaryocytic marker GPIb (not shown). Therefore, part of these cells may represent a megakaryoblastic population, but it is likely that most of them are multipotent hematopoietic progenitors (see below). To search for other cell types, YS cells were labelled with Gr-1 and B220 mAb, which stain granulocytic and lymphoid cells, respectively. No B220^{+} cells and only a few Gr-1^{+} cells (0-5 per 10,000 cells) (Fig. 1C) were encountered in each conditions, indicating either the lack of substantial numbers of lymphoid and granulocytic progenitors in the YS or the inability of committed progenitors to differentiate *in situ*.

Hematopoietic Gene Expression

We monitored the expression of lineage-specific genes by semi-quantitative RT-PCR (Fig. 2). *HPRT* cDNA amplification was used as a control to normalize the cDNA content of the samples and *VE-cadherin* expression to exclude a possible contamination of *VE-cadherin*$^{-/-}$ YS by maternal blood cells. Interestingly, Southern blots of RT-PCR products did not reveal a significant difference in gene expression between wild type and *VE-cadherin*$^{-/-}$ YS at all time points tested (Fig. 2). Strong signals for *αIIb* confirmed the existence of multipotent progenitors and/or megakaryoblastic cells. The existence of a megakaryoblastic population was further substantiated by the expression of *PF4*, a specific marker of the megakaryocytic lineage. The *myeloperoxidase (MPO)* gene was chosen for granulocytic cells as it is found at the promyelocytic stage with no cross reactivity with monocytes. Faint but consistent *MPO* cDNA amplification products could be detected, in agreement with the existence of a small number of Gr-1^{+} cells in the YS. For the erythroid compartment, *βH1* but also the *adult β globin (βmaj)* genes were abundantly expressed. However, the presence of *bmaj* cDNA is not sufficient in itself to indicate that definitive erythroid

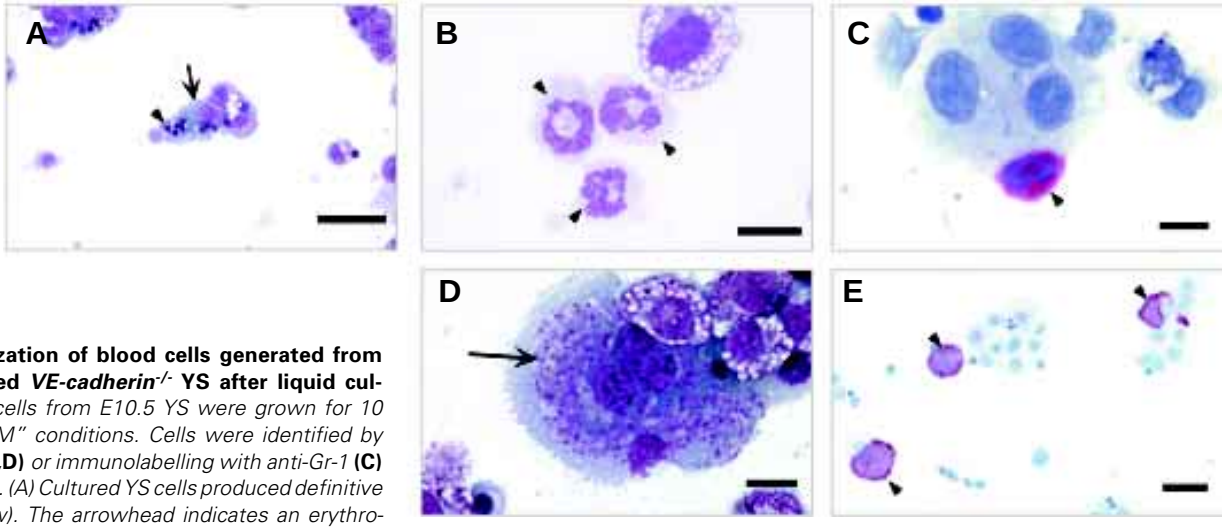

Fig. 3. Characterization of blood cells generated from collagenase-treated *VE-cadherin*$^{-/-}$ YS after liquid culture. *Dissociated cells from E10.5 YS were grown for 10 days under "GEMM" conditions. Cells were identified by MGG staining* **(A,B,D)** *or immunolabelling with anti-Gr-1* **(C)** *or anti-αIIb mAb* **(E)**. *(A) Cultured YS cells produced definitive erythrocytes (arrow). The arrowhead indicates an erythrocyte extruding its nucleus. Numerous neutrophils, (arrowheads in B,C) and megakaryocytes (D, arrowheads in E) were also visible. The arrow in (D) shows a megakaryocyte filled with proplatelet granules. Bars: 50 μm in (A,E), 10 μm in (B-D).*

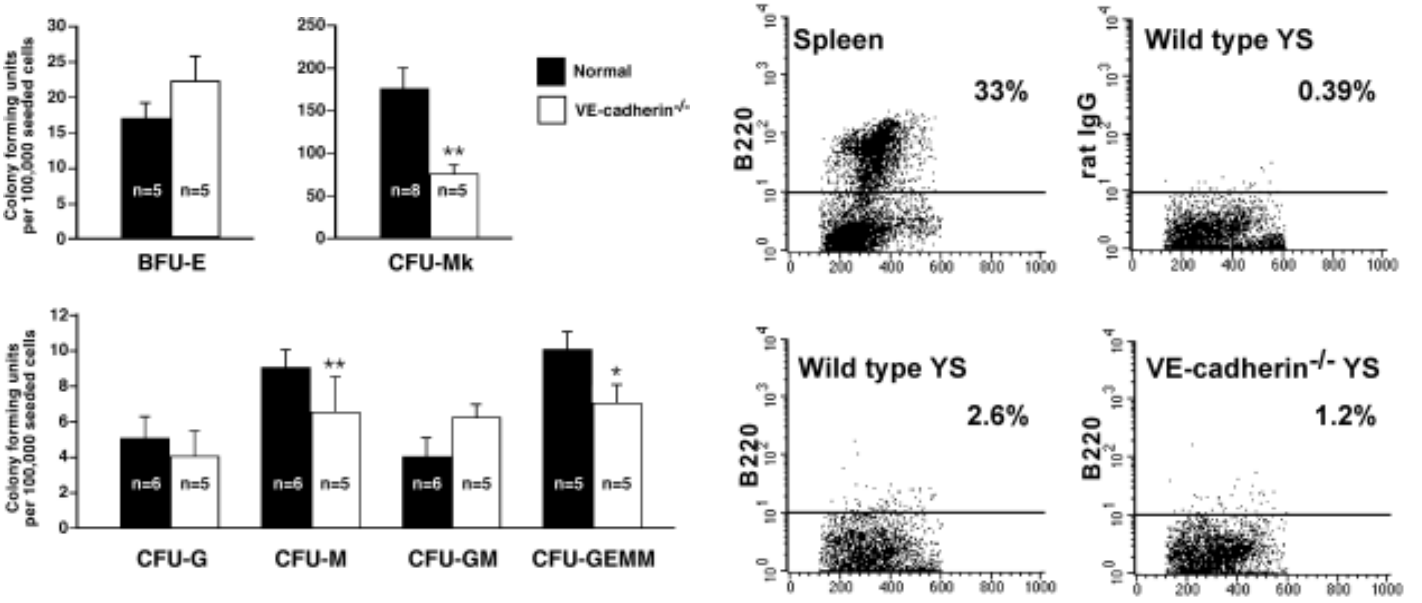

Fig. 4. (Left) Colony formation by various hematopoietic progenitor cell types after clonal culture of E9.5 YS cells. *After collagenase treatment, cells were seeded in triplicate at 10^5 per dish in semi-solid medium (methylcellulose or collagen). Cultures were performed under "GEMM", "BFU-E" or "CFU-Mk" conditions. After 10 days, colonies were identified and scored by visual inspection. CFU of each cell type were obtained from YS of either normal or VE-cadherin$^{-/-}$ mice. Data are the mean (+ SEM) of colonies deriving from five to eight YS, as indicated. Significant differences between VE-cadherin$^{-/-}$ and normal colony numbers are indicated: *, p<0.05; **, p<0.02.*

Fig. 5. (Right) Flow cytometry profiles of E9.5 YS progeny after culture in lymphoid conditions for 10 days. *(Top left) Spleen cells were immunolabelled with B220. (Top right) Wild type cultured YS cells were labelled with non-immune rat IgG. Labeling of wild-type (bottom left) and knockout (bottom right) YS with B220 mAb. The percentage of positive cells is indicated.*

progenitors are generated at this stage, because expression of both genes can be found in single primitive erythrocytes (Palis *et al.*, 1999). Finally, *RAG 2* expression was not observed in YS cells, suggesting that no differentiation occurred along the lymphoid pathway.

Multipotential Hematopoietic Progenitors originate in the YS

The existence of YS hematopoietic progenitors was first demonstrated by a liquid culture system using collagenase treated YS. Figure 3 shows MGG stains and immunolabelling of cells after 10 days of culture of *VE-cadherin*$^{-/-}$ E10.5 YS. In GEMM conditions (see Materials and Methods) the majority of erythroid cells were either enucleated erythrocytes or cells in the process of nucleus expulsion (Fig. 3A), thereby revealing the existence in the YS of progenitors generating mature erythrocytes. After culture, numerous neutrophils were visible on cytospins indicating that the YS does indeed contain granulocytic progenitors (Fig. 3 B,C). MGG and αIIb stains showed large and mature megakaryocytes with polyploid nuclei and the cytoplasm filled with proplatelet granules (Fig. 3 D,E).

To evaluate the number of progenitors in *VE-cadherin*$^{-/-}$ *versus* normal YS, we examined their CFU potentials at E9.5, in three different semi-solid culture assays: "CFU-GEMM", "BFU-E" and "CFU-Mk" (see Materials and Methods). The BFU-E contents were similar (Fig. 4), which suggests that definitive erythroid progenitors located in wild type YS are generated *in situ* with no contribution from the embryo proper. CFU-Mk were abundant in YS but the number of precursors was significantly reduced (p<0.02) in those from *VE-cadherin*$^{-/-}$ YS. Consequently, although confirming the megakaryocytic potential of the YS (Xu *et al.*, 2001), these data do not exclude a possible intraembryonic Mk progenitor contribution in the wild type. Similar numbers of CFU-G and –GM were noticed in both genotypes; however, CFU-M and –GEMM were significantly reduced in *VE-cadherin*$^{-/-}$ YS at E9.5 (p<0.02 and 0.05, respectively). Altogether, these results demonstrate that multipotent hematopoietic progenitors, capable of colony formation *in vitro*, are present in the YS of normal and *VE-cadherin*$^{-/-}$ embryos.

One possible cause for the reduced number of some progenitors in the mutant is the genetic model itself. VE-cadherin deficiency may alter the capacity of endothelial cells to sustain a full hematopoietic program since these cells are known to support developmental hematopoiesis (Fennie *et al.*, 1995; Lu *et al.*, 1996; Auerbach *et al.*, 1998; Ohneda *et al.*, 1998; Xu *et al.*, 1998). Microscopic analyses did not reveal a loss of endothelial cells in *VE-cadherin*$^{-/-}$ YS sections (not shown). To better examine this possibility, we assessed the percentage of endothelial *versus* total YS cells by PECAM immunolabelling followed by flow cytometry. The average proportion of YS endothelial cells was slightly higher in mutant *versus* normal controls [7.54 ± 2.13% (n=4) *versus* 4.65 ± 0.22% (n=3), respectively]. Therefore, the lower number of hematopoietic precursors in *VE-cadherin*$^{-/-}$ YS cannot be attributed to the lack of endothelial cells.

Lymphoid Progenitors of the YS at E9.5

Analysis of lymphoid progenitor content was performed by coculture with S17 stromal cells in conditions allowing growth of B cell progenitors. Flow cytometry profiles of E9.5 YS cells grown under these conditions did not show significant B220$^+$ cells in

either wild type or *VE-cadherin*$^{-/-}$ YS (Fig. 5). Similar results were obtained with E10.5 YS cells (not shown). These data are consistent with those previously obtained by Cumano and colleagues (Cumano *et al.*, 1996) after organotypic culture, and suggest that the YS does not contain lymphoid progenitors at these stages.

Discussion

Using a mouse genetic model in which the YS is not in communication with the embryonic circulation, we could demonstrate that this extraembryonic compartment is a source of adult-type multineage hematopoietic precursors, exclusive of lymphoid cells. These data were established on morphological, phenotypical and genetic criteria. Previous studies, using organotypic cultures of tissues removed before circulation, as well as long-term reconstitution assays of lethally-irradiated mice led to conflicting results (Medvinsky *et al.*, 1993; Cumano *et al.*, 1996; Medvinsky and Dzierzak, 1996; Yoder and Hiatt, 1997; Yoder *et al.*, 1997a; Yoder *et al.*, 1997b; Nishikawa *et al.*, 1998; Palis *et al.*, 1999; Palis *et al.*, 2001; Cumano *et al.*, 2001). An obvious explanation for these controversies may be related to the fact that assays of hematopoietic potential depend upon particular *in vitro* and/or *in vivo* conditions. Hence, explanting tissues and culturing them *in vitro*, or injecting progenitors into adult *versus* newborn hosts, may modify their hematopoietic activity. To our knowledge, this is the first time that an *in vivo* hematological investigation of the YS could be performed at such stage, independently of intraembryonic hematopoiesis.

Although adult multilineage hematopoietic progenitors do exist in the YS, they do not differentiate into mature blood cells, with the exception of macrophages. Altogether, these data show that the YS does not provide an appropriate environment for full hematopoietic differentiation, a situation comparable to the P-Sp/AGM, which supports stem cell emergence but not hematopoietic activity (Godin *et al.*, 1999). In agreement with these findings, definitive blood cells could not be detected in the fetal circulation before liver colonization at E10.5.

Comparison of *VE-cadherin*$^{-/-}$ and normal YS progenitor contents (Fig. 4) showed similarities in BFU-E, CFU-G and CFU-GM amounts. However, CFU-Mk, -M and -GEMM were reduced in number in the mutants. These differences in progenitor content could be explained by one of at least three possibilities. (i) As mentioned above, embryo-derived survival factors may be lacking in the *VE-cadherin*$^{-/-}$ YS. Minimal levels of SCF were detected in the YS whereas fetal liver expresses much higher levels (Matsui *et al.*, 1990). SCF acts on multipotential and progenitor cells to promote survival, proliferation and differentiation (Ashman, 1999). Therefore, SCF may be a limiting factor for any YS hematopoietic activity, when the YS is not in communication with the embryonic vasculature. This may be also true for IL-3, which is considered as one of the most potent stimulators of megakaryocytopoiesis (Majka *et al.*, 2001). As far as we know, embryonic IL-3 expression is not documented but our own data showed very weak RT-PCR amplification signals for this cytokine in YS compared to embryos at E9.5/10.5 (not shown). The megakaryocytic pathway may thus be altered in the mutant. This hypothesis is supported by previous data from Cudennec *et al.* (1981) obtained in another model. Using transfilter organ culture of early YS and fetal liver, the authors showed that YS did not make the growth activities, then known as Burst-Promoting-Activity, and that fetal liver-produced molecules were capable of inducing definitive erythropoiesis in associated YS. (ii) As demonstrated earlier, YS endothelial cells support the maintenance and expansion of early YS-derived hematopoietic stem cells (Fennie *et al.*, 1995; Lu *et al.*, 1996). Furthermore, other authors reported that YS endothelial cells may be the source of vitelline hematopoiesis (Nishikawa *et al.*, 1998). Although we showed that there was no decrease in the endothelial content of mutant YS, one cannot rule out that VE-cadherin deficiency may alter the capacity of endothelial cells to support hematopoiesis by an unknown mechanism. (iii) Although unlikely, some hematopoietic progenitors originating in the embryo may colonize the YS and increase its precursor content. Although these three possibilities remain open, our data exclude a major contribution of intraembryonic precursors in YS hematopoiesis, as previously suspected.

As opposed to other hematopoietic lineages, lymphopoiesis was insignificant in E9.5/10.5 YS. Interestingly, Yoder *et al.* (Yoder *et al.*, 1997a) reported that CD34^{+} E9.0 YS cells repopulated multiple blood cell lineages in newborn hosts, suggesting that YS cells placed in an ectopic environment may acquire a lymphoid potential. Furthermore, data from Nishikawa *et al.* (Nishikawa *et al.*, 1998) demonstrated *in vitro* generation of lymphohematopoietic cells from endothelial cells purified from mouse YS. Paradoxically, these authors found that the hematopoietic progenitors from the YS were not competent to give rise to lymphocytes, suggesting that the YS does not provide an effective environment for the acquisition of lymphopoietic competency during differentiation from endothelial to hematopoietic cells. Our data confirm that the early commitment of lymphoid precursors is blocked in the YS. Nevertheless, our study does not preclude possible lymphopoiesis in the YS at later times.

TABLE 1

PRIMERS USED FOR PCR AMPLIFICATIONS

Gene		Primers	Annealing temperature (°C)	Cycle number	Size (bp)
βmaj	5' :	cac aac ccc aga aac aga ca			
	3' :	ctg aca gat gct ctc ttg gg			
	Probe :	gtg cac ctg act gat gct ga	55	24	578
βH1	5' :	ctc aag gag cct ttg ctc a			
	3' :	agt ccc cat gga gtc aaa ga			
	Probe :	gtc cag gct gcc tgg cag aa	57	25	200
αIIb	5' :	ggc tgg agc aca cct atg agc t			
	3' :	ctc aac ctt ggg aga tgg ctg			
	Probe :	tgg ccc tgg cac tgt gaa tgg	55	26	170
PF4	5' :	cgc tgc ggt gtt tcg agg			
	3' :	tca cct cca ggc tgg tga			
	Probe :	tgt tgt ttc tgc cag cgg tg	55	30	185
RAG2	5' :	cac atc cac aag cag gaa gta cac			
	3' :	ccc atg ctg gga aat cga c			
	Probe :	cca cct ctt cgt tat cca gct act tgc t	60	32	523
MPO	5' :	tcc ttt gtc cac cc tcc tt			
	3' :	ctg ggg tgg cac cat tgg ag			
	Probe :	gag gag agt cta gtc ttg gg	55	35	250
VE-cad	5' :	acg gac aag atc agc tcc tc			
	3' :	tct ctt cat cga tgt gca tt			
	Probe :	cca gct ctg tga gcc tct gc	57	25	200
HPRT	5' :	gct ggt gaa aag gac ctc t			
	3' :	cac agg act aga aca cct gc			
	Probe :	tca cgt ttg tgt gtc att agt g	55	30	248

The fact that all tested BFU-E colonies contained a majority of enucleated red cells indicates that the erythroid progenitors were of the adult type. The absence of primitive erythroid precursors at E9.5 and 10.5 is in agreement with previous findings showing that primitive erythropoiesis occurs synchronously at E7.5; the pool of primitive erythrocyte progenitors being exhausted at E9.0 (Wong *et al.*, 1986; Palis *et al.*, 1999).

In conclusion, we demonstrated the existence of multilineage hematopoietic cells in the YS; whether these cells migrate to the liver and eventually contribute to adult hematopoiesis is not known (as for intraembryonic progenitors). However, experiments with cells taken from the circulation at various stages (Moore and Metcalf, 1970; Palis *et al.*, 1999) suggest that progenitors emigrate from the YS to the liver. The lymphohematopoietic potential of the P-Sp/AGM region is well documented (Cumano *et al.*, 1996; Medvinsky and Dzierzak, 1996). Furthermore, emergence of hematopoietic progenitors was precisely located to the floor of dorsal aorta in birds (Pardanaud *et al.*, 1996; Jaffredo *et al.*, 1998; Jaffredo *et al.*, 2000) and in mice (de Bruijn *et al.*, 2002). Additionally, functional adult repopulating hematopoietic stem cells could be localized in the umbilical and vitelline arteries (de Bruijn *et al.*, 2000a; de Bruijn *et al.*, 2000b). However, *in situ* examination of the hematopoietic potential –full or partial- of these sites is missing to establish a topological and qualitative picture of mouse embryonic hematopoiesis. The individual contribution of each intra or extraembryonic hematopoietic site to adult hematopoiesis remains an important issue in mammalian development.

Materials and Methods

Cell Preparation

Mice heterozygous for VE-cadherin were mated and the day on which a vaginal plug was found was designated 0.5. On days 9.5 to 11.5, pregnant females were killed by cervical dislocation and conceptuses were dissected in PBS. YS were rinsed several times in PBS, to avoid contamination by maternal blood cells, and precise developmental stage was evaluated by somite counting and developmental features of normal embryos. Individual YS were dissociated in a solution of 0.5% collagenase H (Boerhinger Mannheim) and 0.1% DNAse 1 (Sigma) in Dulbecco's modified essential medium (DMEM glutamax I, GIBCO-BRL) containing 20% fetal calf serum (FCS, Biochrom KG). YS were incubated at 37°C for 1 h, with occasional shaking and pipeting. Isolated cells were counted and either cytocentrifuged for 5 min at 1000 rpm in a Cytospin 2 (Shandon) or used in hematopoietic cultures. Bone marrow was isolated from femurs of adult mice. Spleen cells were obtained by collagenase treatment of adult mouse spleen, as described for YS.

Liquid Culture of Hematopoietic Cells

Cells from collagenase-treated YS were seeded at 25,000 cells/well in 96-well dishes and cultured in Iscove modified Dulbecco's medium (IMDM, GIBCO-BRL) supplemented with 15% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin (GIBCO-BRL), 0.05 mM β-mercaptoethanol and appropriate growth factors. For granulo-erythro-myelo-megakaryocytic (GEMM) cultures, the medium was enriched with 100 ng/ml of stem cell factor (SCF), 50 ng/ml Flt3 ligand, 25 ng/ml IL-3, 100 ng/ml murine thrombopoietin (mTPO) and 2 U/ml murine erythropoietin (mEPO) (all purchased from R&D systems). After 10 days, cells were harvested and cytocentrifuged.

Staining of Cytospin Cells

Cells were routinely stained with May-Grünwald-Giemsa (MGG, Biolyon), dehydrated and mounted in Entellan (Merck). When further characterization was needed, indirect immunocytochemistry was performed on cytospins using monoclonal antibodies (mAb): rat anti-mouse Ly-6G (Gr-1), CD45R/B220, CD41 (integrin αIIb) mAb (all purchased from Pharmingen). Cells were fixed 20 min in 3% paraformaldehyde/PBS, rinsed in PBS, saturated in PBS/BSA 2%, incubated 1 h in primary antibody and revealed with an alkaline phosphatase anti-alkaline phosphatase (APAAP) procedure (Dako). Cells were then counterstained with hematoxylin (Sigma) and mounted in Aquatex (Merck). Slides were observed under an Axioplan (Zeiss) to count the number of positive *versus* negative cells in a population of at least 400 cells.

Hematopoietic Colony Assays

For all the semi-solid cultures, cells were plated in triplicate at a density of 25,000 cells/well in 24-well dishes.

Myeloid conditions. Cells were cultured in IMDM containing 1% methylcellulose (Stem Cells) supplemented with 15% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin and 0.05 mM β-mercaptoethanol. Medium was enriched with 85 ng/ml SCF, 40 ng/ml Flt3 ligand, 65 ng/ml IL-3, 85 ng/ml mTPO and 6.5 U/ml mEPO. GEMM-, granulocytic (G)-, monocytic (M)- and granulo-monocytic (GM)-CFU were counted following 10 days of culture under an inverted microscope (Axiovert, Zeiss).

Erythroid conditions. Cells were plated in the same medium as the myeloid cultures except that the growth factors were 6.5 U/ml mEPO, 65 ng/ml IL-3, 85 ng/ml SCF. Burst-forming unit-erythroid (BFU-E) were observed at 10 days of culture. To visualize the presence or absence of nuclei within differentiated erythrocytes, cells were picked, smeared on a glass slide and stained with MGG.

Megakaryocytic conditions. Cells were plated in a collagen gel including medium (Easyclone TM without cytokine, Hemeris) supplemented with 50 ng/ml mTPO, 10 ng/ml IL-3, 20 ng/ml IL-6 (all purchased from R&D systems). After 10 days of culture, collagen gels containing colonies were collected onto glass slides, then dehydrated in seconds using a nylon mesh and absorbant filter cards. Whole mount immunocytochemistry experiments were performed as described (Vittet *et al.*, 1997) using an anti-integrin αIIb mAb followed by APAAP procedure.

Cytofluorometric Analysis of YS-Endothelial Cell Content

Collagenase-treated E10.5 YS cells were labelled with an anti-PECAM (MEC 13, Pharmingen) mAb, followed by a secondary incubation with a fluorescein-conjugated anti-rat immunoglobulin reagent (Jackson Laboratories). Cells were analyzed by flow cytometry using a FACScalibur (Becton Dickinson). Propidium iodide was used for marking and excluding dead cells.

Lymphoid Culture Conditions

Collagenase-treated YS cells were plated in 24-well dishes (four wells/YS) onto irradiated (1000 Rad) S17 stromal cells, as previously described (Cumano *et al.*, 1993). Culture medium was IMDM supplemented with 15% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.05 mM β-mercaptoethanol, 50 ng/ml IL7 (R&D Systems), 50 ng/ml Flt3 ligand and 85 ng/ml SCF. After 10 days, cells were harvested, stained with a fluoresceine-conjugated B220 mAb (Pharmingen) and analyzed by flow cytofluorometry, as described above.

RNA Extraction and RT-PCR Analysis

YS were pooled and RNA was purified by a micro-scale total RNA isolation procedure (5 Prime-3 Prime). Bone marrow RNAs were isolated by Trizol reagent (Gibco-Brl). RT-PCR analyses were performed using exponential amplification conditions as previously described (Vittet *et al.*, 1996). Oligonucleotides used as primers are indicated in Table 1. *Hypoxanthine phosphorybosyltransferase (HPRT)* cDNA amplification was used as control. Reaction products were electrophoresed, transferred onto nylon membrane (Appligene) and hybridized with their respective probes (Table 1).

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