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Hematopoietic potential of the pre-fusion allantois

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Abstract

We previously showed that the fetal component of the placenta has a vigorous hematopoietic activity. Whether this organ is an environmental niche where hematopoietic stem cells (HSC) proliferate and become committed to various lineages, or whether it is also a site for HSC emergence, was left open. This issue can be addressed only if the components that will give rise to the placenta are tested prior to vascularization. The fetal part of the placenta forms through the fusion of the allantois and the chorionic plate around the stage of 7 somite pairs. The allantois, a mesodermal rudiment that provides fetal blood vessels to the placenta, was retrieved before fusion. We found in this rudiment expression of CD41, a known marker of early embryonic hematopoietic progenitors. c-Kit encoding a progenitor specific receptor was also expressed. Significantly, as early as the 1-2 somite stage, the allantois yielded erythroid, myeloid and multipotent clonogenic progenitors, when pre-cultured *in toto* prior to seeding in a semisolid medium. These results provide evidence that the allantois has hematopoietic potential *per se*. Whether this potential also involves the ability to produce HSC is still to be determined.

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Keywords: Allantois; Yolk sac; Mouse; Hematopoietic progenitor; CD41

Introduction

During development, HSC emerge in specific embryonic sites, wherefrom they seed several sequential niches which promote blood cell amplification and diversification. For a few years the yolk sac (YS) was held to be the unique site capable of producing HSC and, as a corollary, was thought to seed the whole blood-forming system (Metcalf and Moore, 1971). However, the commitment of HSC within the embryo proper

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was uncovered in avian chimeras (Dieterlen-Lièvre, 1975; Lassila et al., 1978; Dieterlen-Lièvre et al., 2001) following which a novel site of commitment could be localized in the region of the aorta in both avian (Cormier et al., 1986; Cormier and Dieterlen-Lièvre, 1988) and mammalian (Godin et al., 1993; Medvinsky et al., 1993) embryos. Depending on the developmental stage at which this site was probed in the mouse, it was christened para-aortic splanchnopleura (P-Sp) on day 7.5–10 (E7.5–10) (Cumano et al., 1996) or aorta/gonad/ mesonephros (AGM, E10–11.5) (Medvinsky and Dzierzak, 1996).

In appropriate culture conditions, the P-Sp yields clonogenic hematopoietic progenitor cells (HPC) (Cumano et al., 1996), whereas long-term reconstituting (LTR)-HSC capable of reconstituting normal irradiated mice are detected only later in the AGM (Müller et al., 1994; Cumano et al., 2001). Thus, the

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consensus focused on the aorta and peri-aortic region as the site responsible for the production of definitive HSC. However, we reported since that the mouse placenta is involved in fetal hematopoiesis by disclosing (i) the presence of *in vitro* clonogenic HPC (Alvarez-Silva et al., 2003) and (ii) that of LTR-HSC (Gekas et al., 2005), the latter conclusion being reached in parallel by Ottersbach and Dzierzak (2005). In all these experiments mother and embryos had distinct markers, so that placental cells from maternal and embryonic origin could be identified. Both short-term HPC and long-term HSC were obtained from the embryo-derived component of the placenta (henceforth designated as fetal placenta), establishing this component as a very active fetal hematopoietic organ.

Comparison with other landmarks suggests that placental activity may play a predominant role in the ontogeny of hematopoiesis. Placental hematopoiesis, as detected by *in vitro* assays, is initiated at the 18–20 somite pair (sp) stage (Alvarez-Silva et al., 2003) in parallel with that in the P-Sp (Godin et al., 1995) and precedes by some 24 h the colonization of the fetal liver (Houssaint, 1981). Most importantly, the LTR-HSC output from the placenta lasts longer and is 15 times that in the AGM (Gekas et al., 2005; Mikkola et al., 2005). Thus, the placenta probably contributes more cells than the AGM to the colonization of the organ rudiments where amplification and differentiation occur, in particular the liver.

How crucial is the role of the placenta depends on whether HSC become committed *in situ*. The placenta forms by fusion of the allantois with the chorionic plate. The allantois is a highly vascularized organ that acquires a vascular network from endogenous angioblasts (Downs et al., 1998) and gives rise to the blood vessels of the umbilical cord and fetal placenta. It is thus a likely candidate site for the commitment and production of hematopoietic cells, a hypothesis supported by the fact that, in the avian embryo, the allantoic rudiment produces blood progenitors (Caprioli et al., 1998, 2001).

Mouse allantois and chorionic plate fuse around the 7sp stage (reviewed in Downs, 2002). Aiming to localize the origin of placental hematopoietic cells, we decided to probe the prefusion, pre-circulation allantois for the production of *in vitro* clonogenic progenitors. In order to study the potential of this early structure, we adopted the same strategy (Fig. 1) as in the case of the early P-Sp, where an *in toto* culture period allowing cell interactions prior to cell isolation and clonal seeding was required for progenitor commitment to occur (Cumano et al., 1996).

The data we report hereafter demonstrate that allantoises from embryos ranging from 1 to 6sp (E7.5–E8) have colonyforming potential. Furthermore, the CD41 antigen is expressed by some cells in the allantois before chorioallantoic fusion and before vascularization. This molecule, the α 2b integrin subunit associated with β 3 in a functional complex, earlier known for its expression by megakaryocytes and platelets, was shown by some of us to mark specifically the intra-aortic hematopoietic clusters in the chicken and mouse embryos (Ody et al., 1999; Corbel and Salaün, 2002) and is now considered as a hallmark of embryonic and fetal HPC (Mitjavila-Garcia et al., 2002; Mikkola et al., 2003; Ferkowicz et al., 2003; Emambokus and



Colony counting and follow up (fluo/phase)

Fig. 1. Experimental scheme used to test the hematogenous potential of the prefusion allantois. Al: allantois; Am: amnios; CP: chorionic plate; E: embryo; EPC: ectoplacental cone; YS: yolk sac.

Frampton, 2003; Ferkowicz and Yoder, 2005; Corbel et al., 2005).

Materials and methods

Mice and embryos

Male and female C57BL/6 and C57BL/6 mice transgenic for an enhanced green fluorescent protein (GFP) cDNA (Okabe et al., 1997) were bred in our animal care facilities. GFP/wild-type heterozygote males were crossed to wild-type females. The day on which the vaginal plug was detected was defined as day 0 of gestation (E0). Pregnant females between E7.5 and E8.5 were killed by cervical dislocation. Embryos were staged according to the number of somite pairs (sp). Conceptuses that had received a GFP paternal allele were selected using a stereomicroscope equipped with UV light.

Immunohistochemistry

Conceptuses were fixed whole in 4% (w/v) paraformaldehyde, embedded in gelatin-sucrose, frozen in isopentane at -80° C and sectioned. Antibody staining and immunoperoxidase (HRP) analysis were performed on 10 μ m cryostat sections as previously described (Corbel et al., 1990). Purified Ig from a rat antimouse CD41 monoclonal antibody (mAb, MW Reg 30, BD Pharmingen, San Diego, CA) were used with a donkey anti-rat IgG biotinylated antibody (Jackson

ImmunoResearch Labs, West Grove, PA). Streptavidin-HRP was purchased from NEN Life Science Products, Boston, MA.

CD41 immunochemistry was also implemented on 5-day organ cultures using the same procedure described above.

Isolation of embryonic tissues and 3D organ culture

E7.5-8.5 embryos were dissected from pregnant mice and placed into PBS for somite pair counting. The allantois, YS, ectoplacental cone (EPC) and cephalic half of embryos were isolated. Dissections were performed under a stereomicroscope. The allantois was retrieved first, special care being exerted to obtain it perfectly clean. To that end, the volk sac and the amnios were delicately torn apart with Dumont No. 5 forceps. When this dissection is carried out at prefusion stages, the allantois pops out. It was cut out with Pascheff-Wolff scissors slightly above the attachment point to the primitive streak. With this method allantoic tissue is free of any contaminating yolk sac. Yolk sac and embryo were obtained in a second step. Organs were washed with PBS and transferred to wells of 4-well multidishes (Nunc), each containing 0.5 ml of RPMI 1640 medium (Gibco-Invitrogen, Cergy-Pontoise, France), 15% fetal calf serum (FCS, Gibco-Invitrogen), 1% kanamycin, 1% HEPES, 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol. Allantois and YS were deposited into the culture medium whereas cephalic halves of the embryo and EPC were cultured on isopore membrane filters (0.8 µm ATTP, Millipore) floating on the liquid medium, according to a classical organ culture protocol. In the case of allantois, up to six organs were co-cultured in each well. After 5 days in liquid culture at 37°C (Fig. 1), the tissues were either treated for immunocytology or dissociated with 0.1% collagenase type V (Sigma, St. Louis) in 3% FCS containing PBS during 1-1.5 h at 37°C. Cells were dispersed by drawing them through fine Pasteur pipets and suspensions were passed through a 40-µm nylon sieve. The cells were washed and resuspended in medium with 3% FCS at 4°C.

In some cases, c-Kit and Ter119 expression was investigated by flow cytometric analysis (FACS) using PE (phycoerythrin)-conjugated mAbs against c-Kit (anti-CD117, ACK45), Ter119 and isotype control Ig (BD Pharmingen). Cells were incubated for 30 min at room temperature with mAbs and washed in FCS containing PBS.

In vitro colony assays

Myeloid and erythroid progenitor cells were detected by their colonyforming ability in semisolid cultures (Fig. 1). Cells obtained from 3D-cultured tissues were seeded in 1 ml of Iscove's medium (IMDM) containing methylcellulose medium and recombinant cytokines (Methocult GF M3434) (Stem cell Technologies). Three types of progenitors developed; myeloid colony-forming cells (CFC) that gave rise to macrophage and monocyte (M) colonies; erythroid progenitor cells, i.e., BFU-E on one hand and Ery-CFC (Palis and Yoder, 2001) on the other, that gave rise to erythroid colonies; Mix-CFC that generated mixed colonies containing an erythroid component plus at least two more lineages. Morphological and quantitative screening of the cultures was performed at different time points with an inverted fluo Olympus JX 70 microscope after incubation at 37°C, with 5% CO2 in air.

Identification of hematopoietic colonies

Hematopoietic colonies were scored between 7 and 16 days of growth. Clusters comprising 10 to 40 cells were frequent at 7 days and were taken into account. Colonies contained more than 40 cells and were identified as Ery-CFC, M-CFC, BFU-E and Mix-CFC. In a few experiments, the cultures were maintained until 40 days.

For cytological diagnosis, clusters and colonies were picked, washed out from methylcellulose in PBS and spread onto superfrost glass slides. Cells were stained with May–Grünwald–Giemsa (MGG), according to a standard protocol. Observation and photographs were performed with a Leica DMR microscope.

In some cases, semisolid cultures were stained with a benzidine solution (1 mg/ml benzidine dihydrochloride/1.5% glacial acetic acid/0.15% H_2O_2) detecting dark blue hemoglobin-synthesizing cells in erythroid clusters (Cluster E) or Ery-CFC-derived colonies.

Macrophages were identified by FACS using PE-conjugated Mabs against Mac1 and isotype control Ig (BD Pharmingen).

Results

CD41 in situ expression in non-cultured pre-fusion allantois and yolk sac (E7.5–8.5)

In order to maintain the anatomical relationships between allantois and YS (see Fig. 2A), the embryos were not dissected, which made somite pair counting possible only from the 4sp stage onwards. CD41⁺ cells were found for a certainty in the allantois at the 4–6sp stages. It is likely that these cells were present earlier. The total number of these CD41⁺ cells was small, less than 10 (Figs. 2A, C), usually distributed among several groups whereas in the YS they were more numerous (Figs. 2A, B) as previously shown by *in situ* and FACS analysis (Corbel and Salaün, 2002; Mikkola et al., 2003; Ferkowicz et al., 2003; Ferkowicz and Yoder, 2005). For comparison, in the embryo proper, CD41⁺ cells are found in the intra-arterial clusters, at E10 only, i.e., 36 h later (Corbel and Salaün, 2002).



Fig. 2. CD41 expression in pre-fusion allantois. Transverse section from a 5sp embryo (E8). (A) Allantois (Al) and YS, scale bar=20 μ m. (B) Boxed area in panel A showing a YS blood island with CD41⁺ cells (arrows), scale bar=10 μ m. (C) Higher magnification of the group of CD41⁺ cells seen in the allantois in panel A (arrow), scale bar=10 μ m.

Choice of the culture methodology

The approach we chose (Fig. 1) had previously proved efficient to detect the emergence of HPC in the embryo proper (region of the P-Sp/AGM) prior to vascularization (Cumano et al., 1996), at the developmental step when the allantoic bud was to be probed. In a first culture step, the 3D arrangement of cells was preserved. Seeding of dissociated cells in a semisolid medium then made it possible to obtain clonogenic progenitors. In the case of the P-Sp, when dissociated immediately after

dissection, this region first gave rise to colonies at the stage of 15sp or so (Godin et al., 1995). In contrast, when the 3D culture period was implemented, it was possible to obtain colonies from the P-Sp as early as the 0sp stage. In the case of the P-Sp, a feeder layer of S17 had been used in the 3D culture step. Here in preliminary experiments, the feeder layer did not enhance *in vitro* thriving of the allantois, nor did the addition of cytokines i.e., SCF, IL3, IL11 and/or IL7, so that the explants were simply immersed in a medium containing 15% FCS. Two different lots of FCS were used throughout this study giving the



Fig. 3. Phenotype of allantoises after 5 days in 3D culture. (A) Culture of one allantois obtained at the 2sp stage. Clusters of round cells (arrows) on a stromal network. Phase contrast, scale bar=100 μ m. (B) FACS analysis for c-Kit expression of cells from 5 co-cultured GFP⁺ allantoises obtained at the 1–2sp stage. GFP⁺/c-Kit⁺ cells: blue. Isotype control: pink line. (C–E) CD41 expression in five co-cultured allantoises obtained from the 4sp stage, (C) scale bar=50 μ m. The two groups of positive cells observed in this culture (one visible in panel C) are magnified in panels D and E (scale bar=20 μ m and scale bar=10 μ m, respectively). (F–H) CD41 expression in two co-cultured allantoises obtained from the 14sp stage, (F) scale bar=50 μ m. (G–H) Higher magnifications from panel F: scale bar=10 μ m.

same results. Progenitors could be identified and counted, and the potentials of allantois and YS could be analyzed at various times of development. The anterior half of the embryo, which is known to be devoid of hemogenic potential (Belaoussoff et al., 1998), was used as a negative control and YS as a positive one.

Evidence for hematopoiesis after the 3D organ culture period

GFP⁺ conceptuses were used according to the protocol used earlier (Alvarez-Silva et al., 2003). Allantoises were dissected leaving out the basalmost part of the stalk. Yolk sac and cephalic halves of embryo were also dissected out (Fig. 1).



Fig. 4. Evidence for erythroid progenitor potential in pre-fusion allantois. (A–B) Cell cluster underlain by stromal cells, from one 1–2sp allantois, 5 days in 3D culture, 2 days in clonogenic culture. (A) phase contrast, (B) bright field, the cluster is faintly pink. Scale bar=100 μ m. (C–H) Clusters and colonies developed from 3–4sp allantoises, 5 days in 3D culture, 14–16 days in clonogenic culture. (C–D) 4sp, one allantois. All cells in the cluster are faintly red (C, phase contrast) and GFP⁺ (D, UV light). Scale bar=100 μ m. (E–F) Colonies from eight co-cultured 4sp allantoises. Benzidine staining. A few cells in a cluster (arrows in panel E) and all cells in a colony (F) stain blue. Scale bar=50 μ m. (G–H) Burst-forming unit-erythroid (BFU-E) colony from six co-cultured 3–4sp allantoises in clonogenic culture. Cells in this large, typical colony are well hemoglobinized (G) and GFP⁻ (H). GFP⁺ cells in this field are probably stromal except for rare, round, small cells in the BFU-E periphery presumably immature erythroblasts (arrows). Scale bar=50 μ m.

At these early stages, the allantois is a very small and thin, diaphanous vesicle-like structure, so that one to six whole organs were pooled and cultured together in one well. In contrast each YS and cephalic half had to be divided into 6–8 fragments, which were co-cultured during the 3D culture period. YS rounded up into spheres, whereas cephalic halves and ECP, cultured on a Millipore filter, flattened. In most cases, allantoises aggregated together in the liquid medium and formed either floating spheres or, more often, round, well circumscribed disks (Fig. 3F), as previously described (Downs et al., 1998). In the latter case, round cells clustered above a network of stromal cells were usually very apparent (Fig. 3A). Red cells were not visible under the microscope. We investigated whether erythroblasts were present by FACS analysis of cell suspensions obtained by enzymatic treatment of 1–2sp allantoises cultured for 5 days. No cells expressed the erythroid-specific antigen, Ter119 (not shown). However, 20% of cells from this culture expressed c-Kit (Fig. 3B). The c-Kit-positive cells were GFP⁺.

In situ expression of CD41 was studied at the end of the 5day 3D culture period. The number of positive cells had increased dramatically by comparison with pre-culture allantoises as illustrated in five co-cultured allantoises obtained at 4sp (Figs. 3C–E, compare with Figs. 2A, C). This antigen was not investigated in explants retrieved before the 4sp stage. The number of CD41⁺ cells present at the end of the 3D culture



Fig. 5. Evidence for myeloid and multipotent progenitor potential in pre-fusion allantois. (A–B) Macrophage colonies. 5 days in 3D culture, 21 days in semisolid culture of rudiments obtained at the 6sp stage. 8×10^4 YS cells (A, phase contrast) and cells from 4 co-cultured allantoises were seeded (B, UV light). (C) Huge macrophage colony from one 3sp stage allantois. 5 days in 3D culture, 40 days in semisolid medium. (D) Higher magnification of insert in panel C; UV light. (E) FACS analysis of cells from a single myeloid colony from one 5sp stage allantois after 30 days in clonogenic culture. Mac1 expression (pink). Isotype control (red line). This result is representative of four experiments. (F) Mixed colony from three 5sp stage allantoises cultured for 7 days in semisolid culture. (G) MGG staining of cells from a mixed colony. m: monocyte; M: macrophage; my: myelocyte; Eb: erythroblast; and E: enucleated red cell. Scale bar=10 μ m.

increased strikingly when the allantoises had been retrieved after chorionic fusion (Figs. 3F-H).

In most cases, the YS fragments explanted together gave rise to distinct spheres floating in the culture medium and all contained CD41⁺ cells and abundant well hemoglobinized cells (not shown). Cephalic halves of embryos spread into cell sheets that never exhibited any CD41-positive cells.

Qualitative assessment of allantoic clonogenic progenitors

Cells from 3D co-cultured allantoises were usually seeded together in one well. Although up to six allantoises were cocultured, the number of cells obtained was small, $5-10 \times 10^3$, so that the results were expressed per number of organs cultured rather than according to the number of cells seeded. In the case of YS, enough cells were obtained for counting prior to seeding, $1-15 \times 10^4$ cells obtained from one or several spheres being seeded. In these clonogenic conditions, clusters and colonies always developed from both appendages. In approximately 50% of allantoic cultures, clusters of pink round cells could be observed as early as the 2nd day. These clusters were noticeably associated with elongated cells (Figs. 4A, B).

Clusters and colonies were recorded between 7 and 16 days. Stromal cells were always GFP^+ and, depending on their type and state of maturity, hematopoietic colonies were or were not GFP^+ (Compare Figs. 4C–D with G–H). Two major types of colonies, erythroid and myeloid, developed from allantoises. Benzidine staining confirmed the erythroid nature of clusters and colonies first identified by their morphology (Figs. 4E–F). Among other erythroid colonies developed from pre-fusion allantoises large BFU-E (Fig. 4G) were noticeable. The hemoglobin-positive cells in these BFU-E were GFP negative as expected because enucleated mature red cells cease GFP synthesis, whereas stromal cells were GFP⁺ (Fig. 4H).

Concerning myeloid progenitors, most were typical M-CFC, similar to those obtained from the yolk sac. At 21 days myeloid colonies were spread out and large measuring 3 to 5 mm whether from YS (Fig. 5A) or allantois (Fig. 5B). When M-CFC from pre-fusion allantoises were maintained in culture up to 40 days, they continued growing, some reaching as much as 9 mm in diameter (Figs. 5C–D).

Thirty-day colonies derived from 5sp GFP^+ cultured allantoises were pooled and stained with the macrophage lineage-specific antibody Mac1-PE. Half of the cells expressed the antigen Mac1 (Fig. 5E) in agreement with morphological diagnosis. All cells in the colony depicted were GFP^+ (Fig. 5D). No red colonies survived at this time.

Mix-CFC were also recorded. Most cells displayed morphology and staining characteristics consistent with erythroblastic, erythrocytic and macrophagic phenotypes. Some myelocytes and monocytes were also observed (Figs. 5F–G).

Quantitative assessment of progenitors depending on stage at explanation

Embryonic cephalic halves were used as negative controls. The 3D immersion culture conditions, used for allantois and yolk sac, were inadequate for the tissues therein which had to be cultured on floating millipore filters. No colonies ever developed, even though cell survival was excellent. In contrast, all allantois and YS cultures gave rise to all types of colonies (Table 1).

As seeded allantoic cells were too few to be counted, results are presented as histograms, in which the assortment of colonies is plotted against the number of 3D co-cultured allantoises (Fig. 6). As early as the 1–2sp stage all types of progenitors developed from the allantois. The majority of progenitors were erythroid, some of which differentiated with time from clusters to colonies. Myeloid progenitors were detected and more rarely BFU-E and Mix-CFC (Fig. 6A*, Table 1A). At the stage of 3– 4sp, erythroid and myeloid progenitors increased notably, with a tendency for an enhancing influence of the number of 3D cocultured explants (Fig. 6B).

At the 5sp stage, the number of small erythroid (clusters and Ery-CFC) and macrophage colonies decreased, whereas BFU-E and Mix-CFC persisted. These disappeared at the 6 and 7sp stages (Table 1A). Finally after chorioallantoic fusion (9–12sp), progenitors were mainly erythroid (Ery-CFC) and their frequency was similar to that at pre-fusion stages.

All data reported in Fig. 6 concern colonies recorded between 7 and 16 days of culture. When cultures were pursued longer, both number, size and types of colonies changed. Erythroid clusters recorded at day 7 grew into true colonies constituted by more than 40 cells (Fig. 6B*). The myeloid lineage evolved according to the same pattern (Fig. 6C*). The size of these colonies increased continuously until day 40 where they could be found to express Mac1 (Fig. 5E).

Colony numbers were different between duplicates (Fig. 6D#). This discrepancy indicates that the HPC frequency in prefused allantois is low. Evolution of HPC frequency after fusion (Fig. 6F) needs further investigation.

Table 1

Evolution of clonal progenitor numbers, according to allantois and yolk sac stage at retrieval

(A) Allantois						
Stage (sp)	Number of explants	Clusters E	Ery-CFC	M-CFC	BFU-E	Mix-CFC
1-2	28	107	36	20	2	4
3-4	25	220	144	44	3	6
5	25	113	21	16	6	6
6	46	70	90	13	0	0
7	17	91	24	13	0	0
9-12	15	155	122	10	0	1
(B) Yo	lk sac					
Stage (sp)	Number of seeded cells $(x 10^4)$	Clusters E	Ery-CFC	M-CFC	BFU-E	Mix-CFC
1-2	12.5	16	164	20	1	2
3-4	21	8	25	35	0	0
5	10	0	15	13	6	4
6	76	151	181	75	11	3
7	17	47	4	81	61	62
9-12	19	0	115	62	33	21

Semisolid cultures were scored for clusters and colonies between 7 and 16 days.



Fig. 6. Compared assortment of colonies obtained from allantoises retrieved from various stages. Semisolid cultures were scored between 7 and 16 days. Each group of columns represents colonies obtained from one well. In abscissa: number of allantoises co-cultured (1 to 6). (A*) This co-culture gave rise to different colony types (7 M-CFC, 2 BFU-E and 4 Mix-CFC). (B*) Thirteen clusters here recorded on day 7 had been replaced by erythroid colonies on day 21. (C*) A single macrophage colony was recorded on day 7 and five on day 14. (C⁺) Compare with Fig. 7C⁺. See Results. (D[#]) Eight co-cultured allantoises were split in 2 equal lots (4×10^4 cells each). Ten erythroid colonies developed from each lot, no macrophage colony in one and 7 in the other.

In the YS, as early as the 1–2sp stage, large numbers of myeloid and erythroid progenitors developed (Fig. 7A and Table 1B). At the 3–5sp stage, the number of M-CFC dropped (Figs. 7B, C compare with Figs. 6B, C). At later stages, i.e., from the 6 to 12sp stage, numbers of BFU-E and M-CFC increased steeply becoming more frequent than these obtained from allantoises (Figs. 7D, F compare to Figs. 6D, F).

Altogether, colonies obtained from YS were very numerous. Nevertheless in some cases where an equivalent number of cells from the two organs could be seeded, the HPC potential of the pre-fusion allantois appeared similar to that of YS. For instance, in a case where allantois and YS from the same three 5sp embryos were treated in parallel, 4×10^4 seeded cells gave rise to 13 colonies in the case of the allantoises (Fig. 6C⁺) compared to 16 in the case of the YS (Fig. 7C⁺). The distribution of colony types between allantois and YS was comparable, 3 and 8 M-CFC, 6 and 4 Mix-CFC, 4 and 4 BFU-E, respectively.

To summarize, colonies were obtained from allantois beginning at the earliest stages despite the minute number of cells that could be obtained. Strikingly BFU-E and Mix-CFC developed from the earliest allantois rudiments and apparently dropped from the 6sp stage. Furthermore, the vigorous development of other erythroid and myeloid progenitors, Ery-CFC and M-CFC, definitely indicate that the allantoic bud has



Fig. 7. Compared assortment of colonies obtained from yolk sac retrieved from various stages. Semisolid cultures were scored between 7 and 16 days. Each group of columns represents colonies obtained from one well. In abscissa: number of YS cells cultured: (a) $0.5-2 \times 10^4$, (b) $3-5 \times 10^4$, (c) $7-9 \times 10^4$, (d) 15×10^4 . *See Results.

hematopoietic potential, even if experimentally prevented from fusing with the chorionic plate.

Assessment of ectoplacental cone for clonogenic potential

Fragments of EPC were assessed in parallel with allantois and YS. It appeared that the 3D culture conditions in immersion were inadequate for survival of EPC tissues and thus development of clonogenic progenitors. We later found that the results were strikingly improved when EPC was deposited on a millipore filter floating on the liquid medium, according to a classical organ culture protocol. The preliminary results obtained from 3 experiments, two at 2sp with 6 explants in each (yielding a total of 1 Ery-CFC, 1 BFU-E and 2 M-CFC) and one at 4–6sp with 8 explants (yielding a total of 5 BFU-E and 1 M-CFC), indicate that EPC also displays a clonogenic potential at these pre-fusion stages. These colonies were all GFP+, i.e., not maternal in origin.

Discussion

Allantoic development in the mouse embryo has been precisely described (reviewed in Downs et al., 1998, 2004). This appendage, an outgrowth of caudal splanchnic mesoderm that appears during early gastrulation and participates in the formation of the chorioallantoic placenta, becomes vascularized at about the 7sp stage, fusing to the chorionic plate at the same period (Downs, 2002). In the avian embryo, the allantois was shown to produce hematopoietic progenitors and angioblasts capable of colonizing the bone marrow of a host embryo (Caprioli et al., 1998, 2001). Thus, the question was whether, in the mouse also, endothelial and hematopoietic progenitors (maybe hemangioblasts) become committed in the allantoic bud in situ, but several previous attempts to settle this issue were only partly successful. Whereas vasculogenesis, detected by the differentiation of a VEGFR2 network, occurred in the isolated bud cultured in toto 24 to 72 h (Downs et al., 2001), erythropoiesis was not observed. Downs et al. (1998) found only very few (less than 10) benzidine-positive cells in a small proportion of allantoises from the 6sp stage onwards and concluded that these cells probably were contaminations from the embryo or yolk sac. This number did not increase when allantoises were cultured for 24 to 72 h (Downs et al., 2001). This group also used lacZ transgenic allantoises, whose cells could be traced after grafting into wild-type hosts (Downs and Harmann, 1997; Downs et al., 1998). Erythroid precursors of donor origin were not found in the hosts, whereas in contrast the allantois proved capable of providing endothelial cells. It was then concluded that the allantois had no hemogenic capability, a conclusion sustained by Palis et al. (1999) who also found little or no hematopoietic potential in allantoises isolated from 1 to 16sp embryos. We are now able to uncover evidence of intrinsic hemogenesis.

A difficulty in carrying out the present experiments was that the number of cells obtained from these early allantoic buds was minute, around 5000, precluding the count of cells seeded. One to 6 allantoic buds were co-cultured and quantification was based on the number of buds from which cells were seeded into one well. This method is similar to that implemented in studies about intra-embryonic progenitors where, rather than cell numbers, 'embryo-equivalents' gave the measure of cellular material seeded or injected into irradiated hosts (Kumaravelu et al., 2002; Gekas et al., 2005).

It turned out that colonies were obtained from all stages between 1 and 12sp, and neither the number of colonies nor the range of colony types could be precisely related to the allantoic stage at explantation. Due to limiting availability of cells, the number of progenitors seeded was very small, explaining that, in duplicate cultures, the number and types of colonies were usually different.

Importantly from the earliest time point (headfold stage) clonogenic progenitors were always obtained, provided the allantoic buds had been 3D pre-cultured before dissociation into single cells and seeding in semisolid medium. Thus, it is clear that hematopoietic progenitors become committed *in situ* from the allantoic mesoderm. Compared to the YS probed with the

same method, the allantois also displayed the whole array of colonies. However, during the 3D culture step no signs of erythropoiesis were visible in the allantois, whereas red clusters were prominent in YS cultures. Emergence of progenitors has previously been analyzed in the YS (Palis et al., 1999). We find the first colonies at the same stages as these authors do. At the mid-streak/neural plate stage, the number of the colonies they found was also variable. At later stages they found a large increase in Ery-CFC numbers, which we did not observe. It should be emphasized in this respect that the colonies derived from the YS progenitors present at the time of explantation mature during the 3D culture period of our protocol, as testified by the red color of the explants. Thus, we uncover only the progenitors that become committed after explantation.

Immunocytological CD41 detection, whether performed immediately after dissection or carried out after 3D culture, confirms the intrinsic hemogenic capacity of the allantoic bud. CD41⁺ cells were found by the stage of 4sp and may be present earlier. At 4sp, these cells were scarce, especially when allantois was compared to YS in the same embryo. Thus, two lines of evidence, namely red cell differentiation and CD41, as well as c-Kit expression, indicate that the blood-forming potential of the allantois develops later than that of the YS. When the allantoic buds were 3D cultured, the number of CD41⁺ and c-Kit⁺ cells increased significantly, thus justifying the two-step culture strategy. These cells were Ter119 negative, this antigen being expressed at late stages of erythroid maturation but absent from early progenitors such as BFU-E (Kina et al., 2000). The culture conditions made it possible to detect myeloid, erythroid and multipotent progenitors. Multilineage (lymphoid potential) and LTR potential have still to be analyzed. If further investigations establish the allantois as a site of definitive HSC commitment, it will be clear that the role of this appendage in founding the bloodforming system is primordial. It has indeed been shown that the placenta produces 15 times more progenitors and HSC than does the AGM (Alvarez-Silva et al., 2003; Gekas et al., 2005; Ottersbach and Dzierzak, 2005). The steep increase in HSC populating the fetal liver between E12 and E13 (Kumaravelu et al., 2002), which these authors found difficult to account for, might be due to immigration of HSC present in the placenta.

Our preliminary results indicate that the EPC is also capable of yielding progenitors.

To conclude, the present experiments bring about evidence that hematopoietic progenitors become determined in the allantois before the onset of vascularization. Therefore, the allantois, which provides to the fetal part of the placenta the umbilical artery, vein and endothelial network, may also play a role in seeding the blood-forming system.

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