Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny

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Summary

Quail–chick intracoelomic grafts of organ rudiments were used to study the origin of endothelia and haemopoietic cells during avian organogenesis in conjunction with the monoclonal antibody QH1 which recognizes the quail haemangioblastic lineage. Results differed according to the germ-layer constitution of the grafted rudiments. In the case of the limb buds, endothelial cells from the host invaded the graft through an angiogenic process. Haemopoietic progenitors from the host also colonized the grafted bone marrow.

In contrast, rudiments of internal organs provided their own contingent of endothelial precursors, a process termed vasculogenesis. Nevertheless, haemopoietic cells in these organs were all derived from the host. In the lung, this extrinsic cell population appeared regularly scattered around the parabronchi and had a macrophage-like phenotype. In the pancreas, the granulocytes which differentiate as dense aggregates located in the wall of the largest vessels were extrinsic. Similarly in the spleen, a mesodermal primordium that develops in close association with the pancreatic endoderm, endothelial cells were intrinsic and haemopoietic cells host-derived.

This study demonstrates that, in ontogeny, vascularization obeys different rules depending on which germ layer the mesoderm is associated with: in mesodermal/ectodermal rudiments angiogenesis is the rule; in mesodermal/endodermal rudiments, vasculogenesis occurs. However, in these internal organs undergoing vasculogenesis, endothelial and haemopoietic cells have separate origins.

We put forward the hypothesis that the endoderm induces the emergence of endothelial cells in the associated mesoderm. Formation of blood stem cells may also involve interactions between endoderm and mesoderm, but in this case the responding capacity of the mesoderm appears restricted to the paraaortic region.

Key words: chick, quail, vasculogenesis, angiogenesis, haemopoiesis.

Introduction

By definition, angiogenesis (Hertig, 1935) is the process through which new blood vessels arise from preexisting ones. Extensively analysed in the case of tumour vascularization (see reviews in Folkman, 1985a and b), this process involves attraction and proliferation of endothelial cells into tissues which emit appropriate stimuli. The term of vasculogenesis on the other hand is reserved for the emergence of blood vessels occurring de novo in the early embryo (Houser et al. 1961; Gonzalez-Crussi, 1971; Feinberg & Beebe, 1983; Pardanaud et al. 1987; Risau & Lemmon, 1988). Using QH1, a monoclonal antibody specific for quail endothelial and haemopoietic cells, we have previously described the development of the endothelial network by vasculogenesis in the early quail blastodisc (Pardanaud et al. 1987). A similar analysis was carried out by Coffin & Poole (1988).

Scattered experimental evidence demonstrates that vasculogenesis and angiogenesis can occur as independent processes during ontogeny. Early authors isolated, removed or destroyed regions from avian blastodiscs and concluded that vascularization developed normally in the remaining regions, and thus did not rely on ingrowth of vessels from removed parts (Miller & McWhorter, 1914; Reagan, 1915). They could show in particular that vascularization of the area pellucida did not depend on ingrowth of vessels from the area vasculosa. In quail embryos grafted on a chick extraembryonic area, we also observed that vessel endothelia arose independently in the chick or quail regions of these 'yolk-sac chimaeras' (Beaupain et al. 1979). Our recent observations clearly indicate that QH1-positive cells arise individually according to a time-specific pattern in different regions of the blastodisc and link secondarily into a network (Pardanaud et al. 1987).

On the other hand, angiogenesis also occurs later in
development. These data were established using quail–chick interspecies grafting and subsequent tracing of quail cells by means of the Feulgen-stained nucleolar marker (Le Douarin, 1969). In this way, Jotereau & Le Douarin (1978) reached the conclusion that the whole limb bud, in particular the bone marrow, is colonized by extrinsic endothelial buds. Similarly, Stewart & Wiley (1981) demonstrated that endothelia colonize the brain, while Ekblom et al. (1982) and Sariola et al. (1983, 1984) showed that the kidney is invaded by endothelial buds. These data led to the idea that, while the early vascular tree forms by vasculogenesis, organogenesis involves an angiogenic process (Risau & Lemmon, 1988).

We decided to investigate whether an angiogenic process was responsible for vascularization of all organ rudiments, in particular by comparing the sequence of events in organs with an endodermal versus an ectodermal epithelial component. We were also interested in the relationship of haemopoiesis to blood vessel development. A common precursor for endothelial and haematopoietic cells in the embryo has been hypothesized (Sabin, 1920) and named the haemangioblast (Murray, 1932). In recent years, markers shared by these two cells types have been described, such as monoclonal antibodies MB1 (Péault et al. 1983) and QH1 (Pardanaud et al. 1987). This sharing of an antigenic epitope has been used as an argument for the existence of a common precursor (Péault et al. 1983). A descriptive study of the formation of blood islands in the yolk sac using MB1 also appeared to suggest derivation from the same ancestor (Péault et al. 1988).

One of us has shown earlier that stem cells responsible for definitive haemopoiesis arise in the embryo proper, rather than in the yolk sac (Dieterlen-Liévré, 1975, 1984a). Our experiments carried out in vivo or in vitro on the origin of intraembryonic stem cells have led to the conclusion that the paraaortic region of the mesoderm in 3- to 4-day (E3–4) avian embryos produces blood stem cells. When the aorta and surrounding cells were grafted from the quail into the chick dorsal mesentery, the transplants yielded typical foci of diffuse haemopoiesis (Dieterlen-Liévré, 1984a and b). The potentialities of cells from chick dissociated aortae were also tested in clonal cultures (Cormier et al. 1986; Cormier & Dieterlen-Liévré, 1988). This region of the embryo appeared as a rich source of haematopoietic progenitors. The concentration of progenitors was actually 3 to 4 times higher in aortic wall cell preparations than in bone marrow from newborn chickens. In contrast, cells obtained from E4 embryos deprived of their aorta yielded no colonies.

We undertook the present investigation to pinpoint the relative parts played in ontogeny by vasculogenesis and angiogenesis and their relationship to haemopoiesis. Our aim was to establish the origin of the haemangioblastic lineage in rudiments which are derived from either the splanchnopleura, i.e. mesoderm plus endoderm, or from the somatopleura, i.e. mesoderm plus ectoderm. Interspecies grafting of these rudiments was carried out. Cell origins were traced with the QH1 antibody, whose affinity is restricted to quail haemangioblastic cells. Thus when the host is a quail, positive cells are extrinsic colonizers while, in chick hosts, positive cells arise from the grafted quail rudiment.

Materials and methods

Quail (Coturnix coturnix japonica) embryos and White Leghorn chick embryos were used. Donor embryos were incubated for 48 to 96 h, host embryos 84 h, i.e. stage 20 according to Hamburger & Hamilton (1951). In order to describe quail embryos stages, the criteria of Hamburger & Hamilton’s chick classification were used rather than Zacchei’s table (1961) which is less precise with respect to the early stages of development.

Wing or leg buds were chosen as mesodermal/ectodermal rudiments. When rudiments were retrieved prior to stage 18 HH, the splanchnopleural sheet was separated from the somatopleural sheet by mechanical dissection in magnesium/calcium-free phosphate buffered saline (PBS). To obtain mesodermal/endothelial rudiments, the donor embryo was immobilized left side up by means of two insect needles. The body wall was taken off permitting access to lung, gut, pancreas and spleen rudiments which were severed.

E3-5 host embryos were prepared as follows: eggs were opened through the blunt end and the shell membrane was ripped off after first moistening it with a few drops of PBS. Using two pairs of fine tweezers, the vitelline membrane and the amnios were carefully removed around the right wing bud. The explant of one species, marked with a few grains of sterile animal black, was placed in the egg, near the host embryo of the other species. A longitudinal incision was made along the right wing bud of the host with a sharpened needle. Then the explant was placed on top of the incision and, using the needle and a pair of fine tweezers, it was pushed into the host coelom. The egg was then closed with adhesive Scotch tape and placed at 37°C.

Operated eggs, supervised daily, were incubated for various lengths of time after the graft. The graft in most cases was located on the right side of the body behind the liver, near the lung and the mesonephros. Viscera were cut off and the explant was carefully removed then cleaned in PBS and fixed in Bouin’s fluid for 2 to 3 h (except for the bone rudiments which were fixed one or more days depending on their age of development). Tissues were embedded in paraffin and serially sectioned at 7.5 μm.

Sections were rehydrated in PBS following two toluene baths. The slides were then treated for 20 min with 2–3% new born calf serum (Gibco) in PBS in order to saturate non-specific antibody-binding sites. Monoclonal antibody QH1, directly coupled with FITC, was applied for 45 min at room temperature within a humid chamber. The slides were washed twice in PBS and mounted in PBS. In some cases, a double labelling with Hoechst stain (Bisbenzimide H 33258, Serva Feinhemiochemica) was performed to visualize nuclei. The slides were placed in a 1 mg l−1 solution in PBS for 3 min and then carefully washed with PBS. They were sealed with nail varnish and observed with a Leitz microscope (Dialux 22) equipped with u.v. epiluminescence.

Results

Vascularization of rudiments at the time of transplantation

QH1 monoclonal antibody has affinity for both endothelial and haemopoietic cells in the quail species. The
Figs 1, 2. Normal development of the vasculature in an E 2-5-3 quail embryo. QH1 immunofluorescence. a, aorta; cv, cardinal vein; g, gizzard; l, liver; s, spleen; vv, vitelline vein; w, wing bud. Bar, 100 μm.

Fig. 1. Stage 17HH. Cross section at the wing bud level. The main vessels are present, displaying a QH1-positive endothelium. Some vascular profiles are present at the base of the limb bud (arrows) while the distal part is devoid of positive cells.

Fig. 2. Stage 19HH. Cross section through the trunk with dorsal side on the left. QH1-positive cells have not yet entered the spleen rudiment. The vascular network in the limb bud is still poorly developed. The ventral aspect of the aortic endothelium is conspicuously lined with haemopoietic cells (arrow), a frequent observation at E3–E4.
appearance of these two cell types is very different, so that QH1-positive cells can be readily diagnosed as one or the other type. Obviously the interpretation of the results in interspecies grafts rests on the state of vascularization at the time of transplantation. Normal quail embryos stained with QH1 between stages 17 and 21HH served to monitor the state of vascularization in different rudiments at stages used to obtain donor rudiments. At stage 17 (Fig. 1), the vascular tree comprised the main vessels, aorta, cardinal veins, vitelline veins and perineural vasculature. The base of the wing bud displayed several endothelial profiles while its distal mesoderm appeared unvascularized. The splanchnopleura had not completed the morphogenetic movements closing the gut and the rudiments of internal organs could not yet be identified as buds. Well-developed endothelial profiles were present between the mesodermal and the endodermal layers of the splanchnopleura. At stage 19 (Fig. 2), the gut was well vascularized, while the spleen rudiment contained no positive endothelia. The first endothelial profiles penetrated into this rudiment at stage 21. What should be kept in mind from this brief description is that, apart from the spleen, all the explanted rudiments were already partly vascularized at the time of explantation.

**Haemangioblastic patterns in grafted limb buds**

*(Table 1)*

**Chick rudiments in quail hosts**

All vessels were entirely lined with QH1-positive endothelium, independent of the age at explantation and the age at autopsy of the transplants. A fluorescent network invested the whole explant, in particular connective tissue surrounding cartilage (Fig. 3, 5), perichondrium (Fig. 6), muscular masses (Fig. 7) and feather buds (Fig. 8). Cartilage appeared as avascular islands devoid of fluorescent cells. Apart from the fluorescent network, these explants were pervaded with a great number of isolated QH1-positive cells. The QH1-positive cells located in the connective tissue around the cartilage displayed two different appearances. Round cells, often in groups, bearing a homogeneous surface QH1 labelling, evoked haemopoietic precursor cells. Cells of the other type, which were wheel-shaped and larger, might be macrophages.

In the older explants (E11 to E13), QH1-positive endothelial cells were the first to invade the marrow (Fig. 9). When the marrow was well developed, the whole endothelial network was positive (Figs 9–11). Positive vessels were also present in bone lacunae (Figs 9, 10). Isolated, round positive cells were observed in great numbers in the extravascular compartment of bone marrow (Figs 10, 11): they could be identified as granulocytes on the basis of their coarse intracytoplasmic granules. The vascular compartment contained negative mature erythrocytes and rare QH1-positive cells, devoid of granules (Fig. 11). At the periphery of the marrow, close to the bone, a few elongated very large positive cells were present (Fig. 10). With Hoechst nuclear stain these cells usually appeared plurinucleated, a characteristic feature of osteoclasts (Fig. 12A,B). The bone was lined with a conspicuous monolayer of QH1-negative cells. The osteoclasts could

![Figs 3-8. Haemangioblastic patterns in non-osseous tissue of experimental limb buds. QH1 immunofluorescence.](image)

![c, cartilage; p, perichondrium.](image)

**Fig. 3.** Chick limb bud in quail host (st 18HH until E12). QH1-positive vessels are present in the connective tissue (arrows) and the perichondrium (thin arrow) around the two cartilaginous masses. QH1-positive isolated cells are also observed in the connective tissue (arrowhead). Bar, 100 μm.

**Fig. 4.** Quail limb bud in chick host (st 16HH until E13). The non-cartilaginous tissue is completely devoid of positive cells. Compare with the reverse combination shown in Fig. 3. Bar, 100 μm.

**Fig. 5.** Chick limb bud in quail host (st 18HH until E13). The vascular plexus depicted here is typically observed in the connective tissue between cartilaginous rudiments of bones; fusiform (arrow) or round (arrowhead) isolated cells are also present. The different shapes indicate that the former are endothelial and the latter haemopoietic. Bar, 50 μm.

**Fig. 6.** Chick limb bud in quail host (st 18HH until E13). The perichondrium, well developed at this level, is now invaded by endothelia (arrow). Numerous positive haemopoietic cells are present in the connective tissue (arrowheads). Bar, 50 μm.

**Fig. 7.** Chick limb bud in quail host (st 18HH until E12). Two muscular masses are present, one cross-sectioned (t), the other longitudinally sectioned (l). Delicate positive endothelia are located between and around myotubes. Bar, 100 μm.

**Fig. 8.** Chick limb bud in quail host (st 18HH until E13). Three feather buds are present, two cross-sectioned, one longitudinally sectioned. In their shaft, positive endothelia surround the pulp (arrows). A bud emerges with its vascular ring at the base of the longitudinally sectioned feather. Note that while the smooth muscles of the longitudinal feather are poorly vascularized, positive endothelia and haemopoietic cells are numerous in the connective tissue (arrowhead). Bar, 100 μm.

<table>
<thead>
<tr>
<th>Table 1. QH1-positive cells in grafted limb buds</th>
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<tr>
<td><strong>Chick in quail</strong></td>
</tr>
<tr>
<td>Endothelia</td>
</tr>
<tr>
<td>Wing</td>
</tr>
<tr>
<td>Leg</td>
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† Explanted between stages 16–21HH, examined between E10–E17.  

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Quail rudiments in chick hosts
The great majority of vessels was completely negative in immunofluorescence (Fig. 4). QH1-positive endothelial cells were present but their scarcity was remarkable in these transplants, whether they were wing or leg. The younger the buds at explantation, the less numerous were these positive cells. In most cases, QH1-positive cells were isolated and located at random within the explants; they had an elongated appearance and displayed pseudopods. Exceptionally, QH1-positive cells could be integrated in a chimaeric vessel in particular in buds retrieved beyond stage 18HH. No positive haemopoietic cells or osteoclasts were ever seen in the rudiment.

Fig. 9. Cross-sectioned bone rudiments show three different stages of marrow development (1,2,3). As the marrow completely replaces the cartilage, the haemopoietic cell population becomes more prominent. In the bone lacunae, positive vessels are visible (arrows). In the connective tissue, an artery displays a chimaeric endothelium (arrowhead). Bar, 100 μm.

Fig. 10. In marrow and bone lacunae, thin positive endothelia delineate lumina loaded with negative mature erythrocytes and occasional positive cells. In the marrow extravascular compartment, positive granulocytes are abundant. At the periphery of the marrow, large fusiform positive osteoclasts sit directly against the bone (arrows) or on the other side of a negative cell monolayer (arrowheads). Bar, 50 μm.

Fig. 11. Higher magnification of the marrow. Vessels lined by thin positive endothelia contain negative mature erythrocytes (e). Positive cells (probably erythroblasts) are scarcely present in the lumen of vessels (arrow). The extravascular compartment is filled with positive granulocytes. Bar, 25 μm.

Fig. 12. Double labelling of an osteoclast. (A) QH1 staining: The binucleate positive cell displays pseudopods and is in close contact with bone matrix (b) under the negative cell monolayer lining the bone which is barely distinguishable. (B) Hoechst nuclear staining clearly shows the two nuclei of the osteoclast (arrows) and the nuclei of the QH1-negative cell monolayer (framed in the stippled line). The bone matrix is devoid of nuclei. Bar, 20 μm.

Haemangioblastic patterns in grafted internal organ rudiments (Table 2)

Whereas mesodermal/ectodermal rudiments underwent an all-or-none colonization by immunofluorescent cells, two QH1-positive populations were resolved in mesodermal/endodermal transplants, one extrinsic and one intrinsic.

Chick rudiments in quail hosts

In this combination, endothelia were not labelled; rather, numerous scattered or aggregated cells belonging to the haemopoietic lineage were QH1 positive, with different phenotypes and distributions depending on the rudiment. For instance, in the lung rudiments, most vessels had a QH1-negative endothelium; only very scarce positive endothelial cells could be seen, and they were usually isolated, while positive vascular profiles were small and exceptional (Fig. 13A,B). Regularly scattered positive cells were prominent within the mesenchyme: these cells were round, had a
brilliantly positive cytoplasm and were distributed uniformly everywhere around the parabronchi (Fig. 13A,B). Occasionally they were gathered into large aggregates in the vicinity of blood vessels. These cells were obviously different in shape and size from the few positive endothelial cells. Their host origin, wide distribution and general occurrence in all explants indicate they probably colonize the lung through a regular process.

QH1-negative endothelium lined all vessels in the pancreas as well (Figs 14, 21). Prominent accumulations of round positive cells surrounded the larger vessels. Such cells, closely aggregated, were in most cases restricted to the vicinity of these vessels (Figs 14, 21). In the case of gut rudiments, six out of seven explants displayed a completely QH1-negative endothelial network (Fig. 15). The last graft, transplanted at stage 16HH and harvested at E10.5, exhibited scarce QH1-positive cells inserted in some endothelia; a few small endothelial profiles were also entirely QH1 positive. On the other hand, in all explants a prominent cell population was recognized by QH1 antibody; it was made of round, uniformly marked cells, dispersed in the wall of the gut (Fig. 15). These cells, presumably haemopoietic cells, were rather numerous in two of the explants, in particular at the basal part of the villi. In the five others, haemopoietic cells were less numerous or even scarce.

Quail rudiments in chick hosts
In this combination, endothelia were QH1 positive while no other positive cells were present.

In the lung rudiments, QH1-positive endothelium built a polyhedral network around the parabronchi (Fig. 16A,B). In two rudiments, among the oldest at explantation, a few vessels displayed a QH1-negative or chimaeric endothelium. QH1-positive cells with a haemopoietic appearance were completely absent from all explants. It should be noted that parabronchial endoderm in the quail always appeared weakly QH1 positive.

In pancreatic grafts, the whole vascular network displayed a QH1-positive endothelium (Figs 17, 22). In one rudiment, explanted at stage 21HH, scarce vessels with a QH1 negative or chimaeric endothelium were encountered. No QH1-positive haemopoietic cells were ever present. As previously observed for the lung and pancreas, the differentiated gut tissue, whether gizzard- or intestine-like, possessed an entire complement of vessels with a QH1-positive endothelium. In the gut wall, longitudinal vessels were located as two concentric rings, an internal one encircling the basal part of the villi and an external one at the outside rim of the mucosa. Radial vessels linked the two rings (Fig. 18). In one exceptional graft explanted at stage 21HH, i.e. comparatively late, a few vascular profiles had either a QH1-negative or chimaeric endothelium. In no rudiments were QH1-positive haemopoietic cells observed. When compared to the lung endoderm, it is noteworthy that the gut endoderm is not stained by QH1.

Haemangioblastic patterns in grafted spleen (Table 3)
The pattern of QH1 cell development was similar in this mesodermal organ to that observed for mesodermal/endothelial ones.

Chick rudiments in quail hosts
Whatever the schedule of transplantation, homogeneous results were obtained. Monoclonal antibody QH1 recognized densely aggregated round cells distributed all over the developing organ (Fig. 19A). It was difficult to diagnose these positive cells as haemopoietic or endothelial. However when large vessels, either veins or arteries, were sectioned it was clear that their endothelium was QH1 negative (Fig. 19B).

Quail rudiments in chick hosts
A fine-meshed network appeared QH1 positive in this combination. The overall appearance of this network

### Table 2. QH1-positive cells in mesodermal/endothelial grafts

<table>
<thead>
<tr>
<th>Chick in quail*</th>
<th>Explant number</th>
<th>Quail in chick†</th>
</tr>
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<tbody>
<tr>
<td>Stomach/intestine</td>
<td>- +</td>
<td>7</td>
</tr>
<tr>
<td>Pancreas</td>
<td>- +</td>
<td>4</td>
</tr>
<tr>
<td>Lung</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
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* Explanted between stages 15–18HH, examined between E8–E13.
† Explanted between stages 15–21HH, examined between E9–E17.

### Table 3. QH1-positive cells in spleen grafts

<table>
<thead>
<tr>
<th>Chick in quail*</th>
<th>Explant number</th>
<th>Quail in chick†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelia</td>
<td>Blood cells</td>
<td>31</td>
</tr>
</tbody>
</table>

* Explanted between stages 19–36HH, examined between E6–E15.
† Explanted between stages 17–26HH, examined between E9 and hatching.
was not radically different from that of positive cell aggregates in the reverse combination, i.e. chick into quail (Fig. 20A). However, when profiles of veins or arteries were present in the sections, their endothelium appeared QH1 positive (Fig. 20B). Only a minority of transplanted spleens presented scarce vessels with a QH1-negative or chimaeric endothelium.

From these observations on larger vessels, as well as
from comparison with results concerning other internal organs (i.e. pancreas, Figs 21 and 22), we feel able to conclude that QH1-positive cells in chick splenic rudiments grown in quail hosts were haemopoietic, while in quail rudiments grafted to chick hosts QH1-positive cells correspond to the sinusoidal network.

Discussion

Using quail/chick interspecies transplants and analysing grafted tissues by means of a quail haemangioblastic specific monoclonal antibody, we have been able to distinguish two different processes responsible for endothelial development during organogenesis in the avian embryo. Rudiments composed of mesoderm and ectoderm are sites for angiogenesis, i.e. invasion of endothelial cells or their precursors, while mesodermal/endodermal rudiments undergo vasculogenesis, i.e. differentiation of endothelial cells from self-contained mesodermal precursors. Furthermore, our experiments have uncoupled haemopoiesis from vasculogenesis while also showing that, in the bone marrow, angiogenesis and haemopoiesis both proceed from extrinsic progenitors. Thus, in this tissue, endothelial cells and blood cells may or may not descend from a common precursor.

The origin of endothelia, haemopoietic cells and osteoclasts in the bone marrow has been investigated earlier using the same approach as ours (Jotereau & Le Douarin, 1978). The novel feature in our experiments is that we analysed the derivations of the cell lineages of interest using monoclonal antibody QH1 instead of relying on Feulgen staining of the quail nucleolar marker. With the very sensitive detection provided by the monoclonal antibody, we confirm that endothelial and haemopoietic cells and osteoclasts are all extrinsic to the rudiments. QH1 makes the identification of all these cells extremely precise, whereas Jotereau and Le Douarin found that pericytes and endothelial cells were not always distinguishable from one another on Feulgen-stained preparations and that only 60% of quail osteoclasts displayed the nucleolus-associated heterochromatin condensations. Nevertheless Jotereau and Le Douarin interpreted their observations as indicating an extrinsic origin of osteoclasts and haemopoietic cells and of the whole endothelial network. Theirs was the first experimental demonstration that osteoclasts belonged to the haemopoietic lineage. With QH1 we confirm that the extrapolation made by these authors to all cells of these types was justified. We would like to stress that, in our experiments, the explanations were performed earlier than in Jotereau and Le Douarin's work, at stages 16 to 21 instead of at stages 23 to 25; this approximately one-day difference did not make any difference in the results.

All investigators who have described the development of the limb bud vasculature have clearly shown that a capillary plexus is present as soon as the limb bud begins protruding from the body wall, i.e. stage 17HH (Searls & Janniers, 1971), despite the existence of a marginal zone of mesoderm devoid of capillaries (Romanoff, 1960; Wilson, 1983; Drushel et al. 1985). In our preparations, a few endothelial profiles were already present at stage 16HH. The contribution of these cells to the vascular network of the developed limb was always marginal, if not negligible. The same was true in Jotereau and Le Douarin's experiments in which transplanted limb buds were one day older. Thus it appears that these early colonizing endothelial buds undergo little proliferation in the limb environment. Regression of the limb bud vasculature as cartilage differentiates has been described (Feinberg et al. 1986; Latker et al. 1986; Hallmann et al. 1987). Necrosis of endothelial cells at this period may be involved in this regression. This process would explain the restricted participation of early colonizing endothelial cells in the later vascular network.

Jotereau & Le Douarin (1978) have shown that endothelial cells still colonize a femur rudiment from E8–13 quail embryos or from E9–15 chick embryos grafted into the opposite species, and that both endo-
thelial and haemopoietic cells from the two species are usually mixed in such experimental bone marrows. Our future experiments will aim to establish the relationship between these two cell lineages in the bone marrow and to specify whether the colonization of the limb bud is a continuous process or whether separate waves of endothelial buds grow into the developing limb.

Angiogenesis has also been demonstrated in the case of the brain, the thymus and the kidney. In the case of brain (Stewart & Wiley, 1981), the transplants were obtained from stage 13HH quails and grafted to the coelom of E3 chick embryos. According to the authors, 'the ectoderm and a small amount of mesoderm were left adhering to the brain fragments'. This mesoderm
now report evidence for vasculogenesis in various todermal/endodermal rudiment (thymus) or a purely mesodermal/ectodermal rudiment (limb bud), a mesectoderm, in contrast with lateral plate mesoderm, did not have the potentiality to give rise to endothelial cells detectable in the quail–chick marker system. In the experiments involving the thymus (Le Lièvre & Le Douarin, 1975), the quail mesencephalic neural crest (and neural tube) was substituted for that of the chick host. The mesenchyme of the thymus was derived from the neural crest, i.e. the so-called mesectoderm, in accordance with the origin of the mesenchyme in this whole region; the thymic mesenchyme, including pericytes, was quail. In contrast, endothelial cells were chick, thus derived from the host. This is, to our knowledge, the only clearcut case of the thymus, which undergoes angiogenesis despite the endodermal nature of the thymic epithelium. Le Lièvre and Le Douarin's interpretation according to which the mesectoderm is devoid of endothelium-forming potentiality fits the data. It is also possible that ectoderm exerts a negative effect on the emergence of endothelial cells in associated mesoderm. In that respect, it is interesting to recall that Kessel & Fabian (1987) have described an inhibitory influence of ectoderm from early blastodiscs on mesodermal erythropoiesis.

The hypothesis according to which endoderm plays a primordial role in the emergence of the haemangioblastic lineage in the mesoderm is not new, having been advocated by Wilt (1965) for the formation of blood islands in the yolk sac. Miura & Wilt (1969, 1970) obtained convincing evidence for the positive role played by endoderm on this process in dissociation–reassociation experiments. In the absence of endoderm, only small collections of blood cells formed and they lacked any endothelial envelope. Endoderm exerted its effect on mesoderm in transfilter association, thus indicating a mediation by a diffusible substance. However, Miura and Wilt could not be sure whether this effect was a true induction or a trophic effect.

In the chimaeric splanchnopleural rudiments, surprisingly, endothelial cells were intrinsic and haemopoietic cells were not. This is particularly striking in the case of the spleen, a truly haemopoietic organ in the avian embryo, that harbours 10 times more monocyte/macrophage progenitors (M-CFC) than the newborn bone marrow at the peak of its activity from E11 to E13 (Yassine & Dieterlen-Liévre, unpublished). Another of the splanchnopleural anlagen which becomes colonized by extrinsic blood cell progenitors is the pancreas, an active granulopoietic organ during chick development (Benazzi-Lentati, 1932; Dieterlen-Liévre, 1965). The spleen and pancreas rudiments both develop in the dorsal mesentery, in which haemopoietic stem cells emerge from E3 to E5 (Dieterlen-Liévre & Martin, 1981; Dieterlen-Liévre, 1984a; Cormier et al. 1986;

Two comments should be made concerning the lung. The first concerns the haemopoietic cells that invade it. The lung is considered as a site of local immune responses (reviewed in Bienenstock, 1984). Macrophages are a particularly prominent population in the immune pulmonary functions (Winkler, 1988). The QH1-positive cells that settle in the chick embryonic lung grafted to the quail probably belong to this population, because, at the time they are observed, seeding of lymphocytes in secondary lymphoid organs has not begun. Future analysis will be applied to the characterization of these cells. The other comment concerns the fact that the quail pulmonary endoderm appears weakly QH1-positive. A related observation has been made with monoclonal antibody MB1 which has a similar affinity for the quail haemangioblastic lineage (Péault et al. 1983). In chimaeric bursa of Fabricius, it was found that when a non-lymphoid quail bursal rudiment was grafted into a chick host, the bursal endothelium started expressing MB1 reactivity precisely when colonizing B progenitors came in contact with it (Belo et al. 1985). It might be worthwhile to investigate whether QH1 reactivity in the lung endoderm is related to the entry of haemopoietic cells.

Since the endothelium-forming capacity of the mesoderm depends on its germ layer association, it is tempting to speculate that growth factors synthesized, respectively, by the endoderm and ectoderm may be responsible for vasculogenesis or angiogenesis. An endothelial cell growth factor related to human aFGF has been isolated from embryonic and adult chick brain (Risau, 1986; Risau et al. 1988). Future research will focus on the potential involvement of this and other growth and chemotactic factors in vasculogenesis and angiogenesis.

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References


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