Induction of vasculogenesis and hematopoiesis in vitro

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Summary

Despite a large number of investigations of embryonic vascular development, in particular in avian embryos, the conditions under which the endothelial and hematopoietic cell lineages emerge remain unknown. As we demonstrate here, both endothelial and hematopoietic cells can be induced by treatment of dissociated quail epiblast with fibroblast growth factors in vitro. These cells aggregate in characteristic blood islands. In long-term culture, the induced endothelial cells gave rise to vascular structures in vitro, i.e. vasculogenesis. No induction was observed in the absence of fibroblast growth factors, and other growth factors like TGF-β, TGF-α and EGF were not capable of inducing blood island formation. Thus, the dissociated quail epiblast provides a remarkably simple test system to investigate cell lineage diversification in higher vertebrates.

Key words: vasculogenesis, hematopoiesis, mesoderm induction, endothelial cell, FGF, avian embryo.

Introduction

In the avian embryo, all mesodermal cells emanate from the epiblast by invagination through the primitive streak (Bellairs, 1986). Two of the first cell types to diversify in the early mesoderm are blood cells and endothelial cells (Gonzalez-Crussi, 1971). Due to their close spatial association in blood islands during differentiation, these cells are thought to be descendants of a common hypothetical precursor, the hemangioblast (His, 1900). Hematopoietic precursor and endothelial cells are capable of differentiating independently of the formation of axial mesodermal structures, such as notochord and muscle (Azar and Eyal-Giladi, 1979). In *Xenopus*, the species in which mesoderm induction has been studied most intensely, the experimental induction of dorsal axial mesodermal structures has been achieved by polypeptides of the TGF- growth factor family, while the ventral nonaxial mesodermal structures, such as blood islands, could be induced by treatment with FGFs (Slack et al., 1987; Smith, 1989). The disadvantage of the *Xenopus* model, however, is that the ectodermal cap is a coherent tissue that always gives rise to a complex embryoid body when induced to form mesoderm. Thus, cell-cell interactions occur in this lower vertebrate system, which prevent the analysis of direct effects of inducing factors.

We are interested in the mechanisms by which vasculogenesis and hematopoiesis are regulated in the avian embryo, because in this system vascular development is best examined and understood. Direct induction of hematopoietic and endothelial cells from avian epiblast has so far been thought not to be possible since these cells were believed to be already committed to form nonaxial mesoderm prior to gastrulation (Mitrani and Shimoni, 1990). This was essentially the conclusion reached from experiments using intact chicken preincubation blastodiscs that were prevented from invaginating (Azar and Eyal-Giladi, 1979; Zagris, 1980): under those conditions epiblast gives rise to blood islands by so called polyingression. Thus, a premesodermal, uninduced condition was believed not to be available in the avian embryo.

Materials and methods

Blastodiscs of stages X to XII (according to Eyal-Giladi and Kochav, 1976) were removed from unincubated quail eggs in PBS and carefully cleaned of adhering yolk and subgerminal cavity. They were then transferred to culture medium (Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics) and mechanically dissociated by gentle pipetting. After resuspending in fresh medium, cells were plated onto 96-well plates at a density of about 0.6 blastodisc per well or onto collagen-coated Lab-Tek™ slides at density of 0.75 blastodisc per well. Human recombinant bFGF, aFGF, EGF (Progen, Heidelberg, FRG), human platelet-derived TGF- 1 (Biermann GmbH, Bad Nauheim, FRG) and TGF- (Promega; Serva, Heidelberg, FRG) were added at varying concentrations: from 1ng to 100 ng/ml final concentration (bFGF, TGF- 1 and TGF-) or up to 1000 ng/ml (aFGF and EGF). Negative controls were grown without factors. For time-response studies either medium containing 100 ng/ml bFGF was replaced by factor-free medium at varying times of incubation or bFGF was added at varying intervals after culture onset to a final concentration of 100 ng/ml. For indirect immunofluorescence, cultures were fixed in 4% formaldehyde for 15 minutes, permeabilized in 100% methanol for 5 minutes and exposed to the QH-1 monoclonal antibody (ascites, diluted 1:200) for 45 minutes or to the MB-1 antibody (diluted 1:10). Binding of the

antibodies was visualized by a TRITC-conjugated rabbit anti-mouse IgG. The presence of endothelial cells in the cultures was further substantiated by incubation with 10 μg/ml DiI-labelled ac-LDL (Paesel and Lorey, Frankfurt, FRG) for 4 hours at 37ºC. For histology of induced blood islands, cultures grown on Lab-Tek™ slides were fixed in 6% glutaraldehyde in cacodylate buffer at pH 7.4 for at least 24 hours at 4ºC. Specimens were embedded in Durcupan® and serial sections of 1 μm were stained with 1% methylene blue.

Results and discussion

In our experiments, we used unincubated quail blastodiscs of stages X-XII (according to Eyal-Giladi and Kochav, 1976) because firstly at these stages no mesoderm is present (blastodiscs consist only of two layers, the epiblast and the hypoblast (Kochav et al., 1980)) and secondly both hematopoietic and endothelial cells of the quail embryo can be identified with the aid of the monoclonal antibodies QH 1 and MB 1 (Pardanaud et al., 1987; Péault et al., 1983). The blastodiscs were dissociated and cultured in vitro (Fig. 1A). Under these conditions endothelial cell differentiation does not occur (Yablonka-Reuveni, 1989). Our results confirmed this previous finding: within 2 days, cultures gave rise to an epithelial monolayer consisting of small epithelial cells with numerous yolk granules (Fig. 1A, inset). During a culturing period of 4 weeks, no endothelial or hematopoietic cells could be found. In contrast, after treatment of the dissociated blastodiscs with 100 ng/ml basic fibroblast growth factor (bFGF), the emergence of characteristic blood island-like cell clusters was observed in all (100 %) epiblast cell cultures after three days. These islands lay on the epithelial monolayer, which developed as in controls, and were found at a mean frequency of 14 per culture well. The islands comprised round cells in their centre and spindle-shaped cells at their periphery (Fig. 1B): the round cells reacted with both the MB-1 and the QH-1 antibody (Fig. 1C, D). Nonspecific peroxidase reactivity was detected in the round and the spindle-shaped cells of the islands indicating that these islands were erythropoietic islands (not shown). Giemsa's staining revealed characteristic hematoblastic cells within the islands (not shown). Moreover, within and adjacent to the islands, flat cells with lamellopodia and fine filopodia were identified as endothelial cells by their immunoreactivity for the MB-1 and QH-1 antibodies (Fig. 1E, F). These cells were integrated in the epithelial monolayer. In semithin sections the islands exhibited the typical morphology of embryonic blood islands that lie on the surface of the epithelial monolayer (Fig. 1G). At the fourth day, blood islands began to disintegrate and blood cells covered large portions of the culture wells. After 6 days blood cells had disappeared from the cultures. After 4 weeks the cultures consisted exclusively of endothelial cells as shown by the immunoreactivity for the QH-1 antibody and uptake of the endothelial cell marker DiI-ac-LDL (Voyta et al., 1984) by 100% of the cells (Fig. 2). In such cultures these cells were capable of forming capillary-like vascular networks when grown to confluence, even on plastic culture dishes in normal tissue culture medium (Fig. 3A). The endothelial strands extended
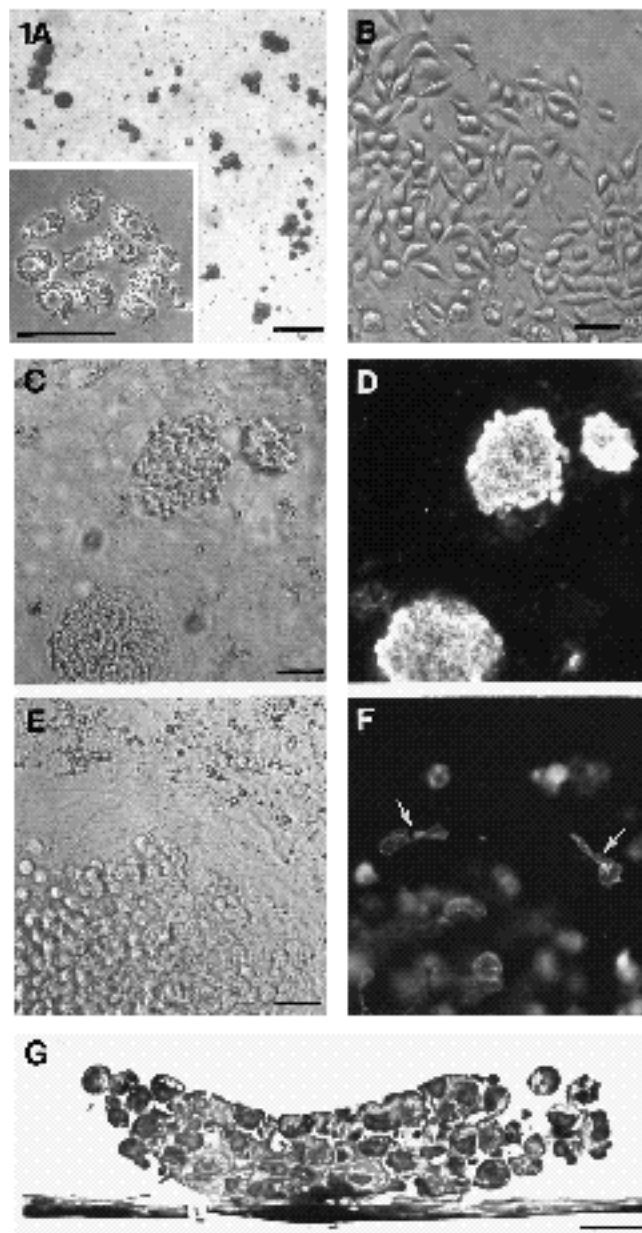

Fig. 1. (A) Dissociated cells and cell clusters of preincubation quail blastodisc immediately after being plated. These cells formed a monolayer of epithelial cells within 2 days (inset in A). In cultures to which 100 ng/ml bFGF had been added blood islands that contained typical spindle-shaped erythrocytes at their periphery appeared 3 days later (B). (D) Immunofluorescence photomicrograph of three blood islands grown in a culture of dissociated quail blastodiscs 3 days after treatment with 100 ng/ml bFGF. Cells were labelled with the QH-1 antibody which specifically recognizes endothelial and hematopoietic cells of the quail. Identical results were obtained using the MB-1 antibody. In the periphery of the blood islands, single QH-1-binding endothelial cells (arrows) were found within the epithelial monolayer of the culture (F). (C, E) Phase-contrast views of D, F: erythrocytes in the periphery of the islands, as shown in B, were dispersed by washing the cultures for immunocytochemistry. (G) Semithin section through a blood island grown under the same condition as those shown in C-F. Scale bars, 200 μm (A), 20 μm (B, E, F), 50 μm (C, D), 10 μm (G).

filopodia and possessed a lumen (Fig. 3B). In controls only non-immunoreactive epithelial cells were seen, which did not take up DiI-ac-LDL.

Formation of blood islands occurred in a dose-dependent manner (Table 1). Maximal induction was achieved at 100 ng/ml bFGF. Identical dose response was recorded for the induction of endothelial cells. Exposure of fresh blastodisc cultures to 100 ng/ml bFGF for a period of at least 7 hours resulted in blood island formation in 100% of the cultures. The responsiveness of the epiblastic cells to bFGF continued for 24 hours after culture commenced. After this time, induction of blood islands was no longer possible (Table 2). Acidic FGF was also effective in blood island induction, but tenfold higher concentrations than those of bFGF were needed, which is consistent with the lower biological activity of acidic FGF in other in vitro systems. In contrast, after treatment with varying concentrations of transforming growth factors 1 and , formation of blood islands was not observed. However, the possibility of synergistic effects by FGF and TGF- , as found in the *Xenopus* mesoderm induction model, remains to be tested (Kimelman and Kirschner, 1987).

In cultures exposed to epidermal growth factor (EGF), single blood islands appeared in 10% of the cultures. In contrast to experiments using FGF, this effect showed no dose dependence and the frequency of islands never exceeded 1 per well. This phenomenon may be explained by the proliferative effect of EGF on the epiblastic cells
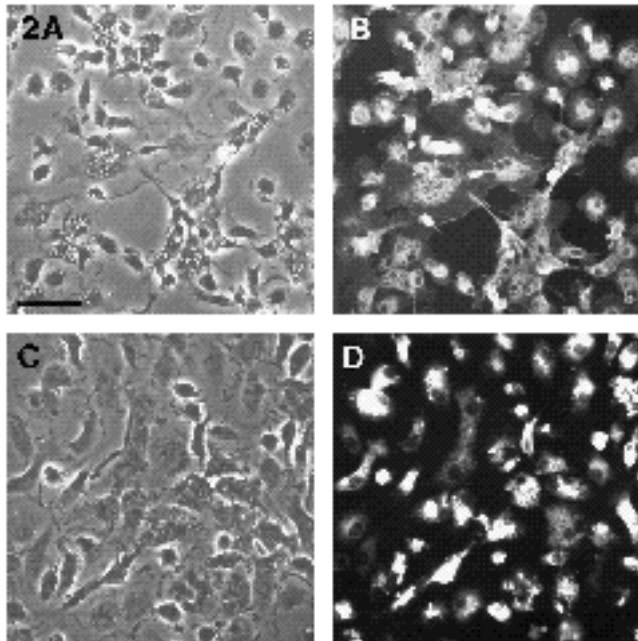

Fig. 2. Fluorescence micrographs of a culture of dissociated quail blastodiscs in the presence of 100 ng/ml bFGF 4 weeks after culture commenced. Exclusively QH-1 positive cells were present (B). After incubation with DiI-ac-LDL 100% of the cells took up the fluorochrome-conjugated ac-LDL (D). These results strongly indicate that the cultures consisted exclusively of endothelial cells. Control cultures underwent dramatic cellular regression and only single epithelial cells remained after 4 weeks. (A, C) Phase contrast of B, D. Scale bar, 50 μm.

Table 1. *Dose response of dissociated epiblast to bFGF*

	Concentration of bFGF in the culture medium (ng/ml)					
	1	5	10	50	100	0 (negative controls)
Wells containing blood islands/ total no. of wells	0/12	1/12	6/12	8/12	12/12	0/62

Methods were as described in "Materials and methods". Cultures on 96-well plates were evaluated under an inverted microscope. Blood islands appeared at the third day and were present for further 3 days. Then, the blood cells disappeared. In tests with bFGF concentrations <50 ng/ml, the number of blood islands was 2 per well on average. At 50 and 100 ng/ml bFGF 14 blood islands per well were counted on average. Endothelial cells were only detected if blood islands were induced by FGF.

that leads to a high cell density followed by spontaneous differentiation of blood and endothelial cell precursors. Nevertheless, the formation of hematopoietic and endothelial cells cannot be regarded as an intrinsic capacity of epiblastic cells, because in control experiments those cells were never found to originate from dissociated epiblastic cells. These observations underline the strong inductive effect of FGFs on the dissociated epiblast, because only FGFs were capable of eliciting the emergence of a high frequency of blood islands and endothelial cells.

We have proposed that at least two distinct mechanisms may be involved in the formation of blood vessels (Risau et al., 1988): angiogenesis, the sprouting of capillaries from preexisting vessels as in tumor and embryonic brain angiogenesis (Risau, 1986), and, vasculogenesis, the development of blood vessels from in situ differentiating endothelial cells as in yolk sac and lung vasculogenesis (Pardanaud et al., 1989). Since the capillary network observed in our cultures developed from endothelial cells that had differentiated in situ from epiblast by the inductive influence of bFGF, this provides an in vitro model system for vasculogenesis; in contrast to angiogenesis in vitro, i.e. the reorganization of capillaries in vitro from disintegrated vascular endothelium (Folkman and Haudenschild, 1980). Endothelial cells induced by FGFs possessed the intrinsic capacity to form a network of capillaries in vitro. Hence, the ability of endothelial cells once induced

Table 2. *Responsiveness of dissociated epiblast to bFGF*

	Culture period before application of 100 ng/ml bFGF (hours)							
	8	17	19	21	24	48	72	0 (positive controls)
Wells containing blood islands/ total no. of wells	8/8	5/6	2/6	0/6	0/8	0/8	0/8	22/22

Methods were as described in "Materials and methods". Cultures on 96-well plates were evaluated under an inverted microscope.
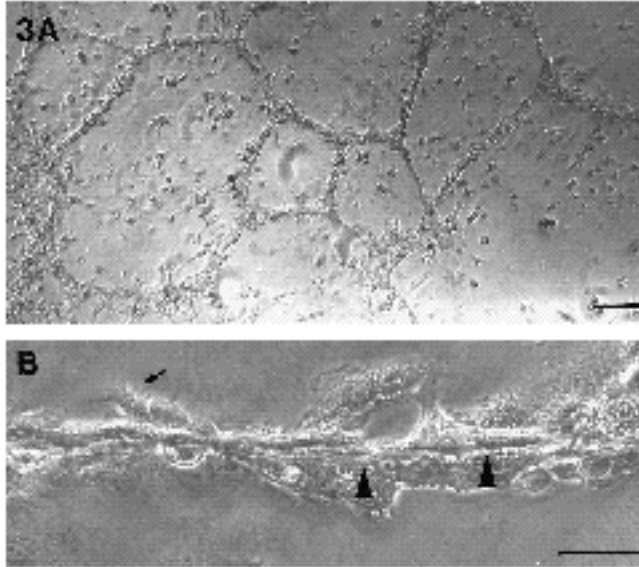
Fig. 3. Vasculogenesis in vitro in an endothelial cell culture (passage 1) derived from dissociated quail blastodiscs. Endothelium was induced by 100 ng/ml bFGF. (A) The endothelial cells formed a network of strands. (B) The strands possessed a lumen (arrowheads) and process-bearing cells at the their border (arrow). Scale bars, 200 μm (A), 50 μm (B).

to form blood vessels is not dependent on additional cues from the environment. This fact strongly supports our concept of vasculogenesis.

Our results raise the important question of what type of cell in the epiblast is induced by the FGFs to differentiate into blood and endothelial cells. In a recent study, epiblastic cells that contributed to the mesoderm by invagination were found to express the HNK-1 epitope, an epitope which is shared by human natural killer cells and embryonic neural crest cells (Stern and Canning, 1990). Among these cells the blood and endothelial cell precursors responsive to FGFs are to be expected. It can be inferred from our time-response studies that these cells possess FGF receptors, and that the responsiveness to FGF is downregulated or completely lost after 24 hours in culture. Since an exposure for 7 hours is sufficient to induce blood island formation, it follows that these receptors either did not have sufficient contact with their natural ligand at the time of explantation or insufficient amounts of this ligand were present. Otherwise spontaneous blood island formation would be expected to occur in the cultures of dissociated blastodiscs.

The concentrations of FGFs needed for blood island induction in vitro are similar to the concentrations needed for mesoderm induction in the *Xenopus* ectodermal cap assay (Slack et al., 1987). The nature of the in ovo signal initiating the differentiation of blood islands remains to be identified, however. Transcripts encoding proteins with homology to bFGF were found in the *Xenopus* oocyte (Kimelman et al., 1988) and in the chicken preincubation blastodisc (Mitrani et al., 1990). Other embryonic factors belonging to the FGF family such as FGF 5 (Hébert et al., 1991) and XeFGF (Isaacs et al., 1992) are also candidates for the natural inducing factor. These may be present as maternal mRNA or as proteins stored within the yolk (Seed

et al., 1988). Further candidates are as yet uncharacterized endoderm-derived factors that were found to stimulate blood island formation (Miura and Wilt, 1969) and possibly may be involved in the regulation of extraembryonic vascular growth (Flamme, 1989).

The culture of dissociated preincubation quail blastodiscs provides the possibility to identify the natural inductive signal. Furthermore, if there exists a common precursor of hematopoietic and endothelial cells, the identification and characterization of this hemangioblastic stem cell seems now to be possible and is a crucial step towards the understanding of the molecular mechanisms that lead to the differentiation into hematopoietic and endothelial cells.

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