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DEVELOPMENTAL ANGIOGENESIS: QUAIL EMBRYONIC VASCULATURE

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Abstract

We have examined the segregation and early morphogenesis of the embryonic vasculature by using a monoclonal antibody for immunofluorescence and by scanning electron microscopy. This antibody labels the presumptive endothelial cells (PECs) as they segregate from mesoderm. Similar embryos prepared for SEM revealed finer details of how these segregated cells interact to form the rudiments of the major blood vessels. Here we concentrate on the development of the dorsal aortae and the posterior cardinal veins. The dorsal aortae form from single PECs which segregate from the lateral mesoderm and aggregate into a loose cord ventral to the somites. These cells become more closely associated and a lumen forms. The posterior cardinal veins form from a loose plexus of cells segregated from the lateral mesoderm on its dorsal surface. These cells become intimately associated with the Wolffian ducts.

KEY WORDS: Endothelium, vascular development, quail, blood vessels, antibodies, morphogenesis.

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Introduction

Early in this century the origin of embryonic vascular endothelium was debated. One side believed these cells originated in the extra-embryonic yolk sac and invaded the embryo and the other side thought they arose locally within the embryo. The morphology of the embryonic vasculature was investigated extensively by injecting opaque liquids like ink (Evans, 1909). The disadvantage of these classic studies was that only patent vessels could be visualized. This did not reveal much about the early segregation and organization of these vessels. By isolating fragments of chick embryos, Reagan (1915) was able to show that blood vessels of the embryo originate within the body proper, not by invasion from the highly vascular embryonic yolk sac.

Meier (1980) and Hirakow and Hiruma (1981) were the first to use scanning electron microscopy to look at early blood vessel formation. The first major blood vessels, the dorsal aortae and posterior cardinal veins, form *in situ* by the segregation from the mesoderm of mesenchymal cells. They observed single cells segregating from the mesoderm and joining into loose cords or plexuses to form the primordia of the major embryonic blood vessels. They could not identify with certainty, however, which cells were destined to become endothelium in the earliest stages. We have reexamined early embryonic vasculogenesis taking advantage of a monoclonal antibody, QH-1 (Pardanaud et al., 1987), which labels quail endothelium and other cells of the hematopoietic lineage. This antibody was obtained by immunizing mice with bone marrow cells obtained from 12 day quail embryos. The specificity of this antibody resembles another monoclonal antibody, MB-1 obtained by immunizing mice with the quail μ chain from plasma (Peault et al., 1983). Some of the labelling studies have been previously presented in abstract form (Coffin and Poole, 1986).

Materials and Methods

Fertile quail (*Coturnix coturnix japonica*) eggs were supplied by the Cornell University Poultry Science Department. For SEM, embryos

were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.15M cacodylate buffer, pH 7.4. Endoderm or ectoderm was peeled off with the aid of fine forceps and sharpened tungsten wire. The embryos were postfixed in 1% osmium tetroxide (in 0.1M cacodylate buffer, pH 7.4) and critical point dried from liquid carbon dioxide after passage to absolute ethanol. After sputter coating with gold/palladium, the samples were photographed on a Hitachi S-520 at 20kV.

Embryos for antibody labelling as whole mounts were fixed overnight with 10% formalin in phosphate-buffered saline (PBS). After washing in PBS, embryos were permeabilized in cold absolute methanol in 3 changes with constant agitation for 3 h. The permeabilized embryos were rehydrated in a graded ethanol series, rinsed in PBS and preincubated overnight in a 3% solution of bovine serum albumin in PBS. Embryos were incubated for 12 h in a 1:200 dilution of QH-1 ascites fluid, rinsed in PBS and incubated with fluorescein-conjugated goat anti-mouse immunoglobin (Cappel, diluted 1:200), dehydrated and mounted in Entellan mounting medium (VWR). They were photographed on a Leitz Orthoplan microscope with epifluorescent illumination.

Results

Figure 1a is a scanning electron micrograph (SEM) of a 3 somite quail embryo with the endoderm removed. Scattered single cells can be seen just lateral to the somites and a plexus further laterally. Figure 1b is an immunofluorescent picture of the same stage embryo showing that these cells stain with the monoclonal antibody, QH-1, and are presumptive endothelial cells (PECs). Figure 2 shows a similar area of a 5 somite embryo revealing that in just over 3 h many more PECs have segregated from the mesoderm. The cells adjacent to the somites soon become organized into loosely associated cords, the rudiments of the dorsal aortae. Figure 3a shows the dorsal aorta rudiment and an extensive plexus which has formed in more lateral regions of a 6 somite embryo. Figure 3b is the corresponding antibody image. Shortly thereafter, the dorsal aortae enlarge and acquire a continuous and sealed off lumen. Figure 4a is a SEM of a 7 somite embryo. The dorsal aorta on the left side is a broader, more continuous cord. In Figure 4b is a 7 somite embryo labelled with QH-1 showing the organization of the dorsal aortae and their relation to the continuous plexus more laterally. Figure 5 is the dorsal aorta of another 7 somite embryo at higher magnification. When the dorsal aorta is fractured and looked at in cross section (Figure 6), one observes not a single lumen but many small ones with thin processes in between. Soon after it becomes possible to fracture open the dorsal aorta to look at its luminal surface. In this example (figure 7) from a 9 somite embryo the beginnings of sprouts forming the intersomitic arteries are clearly visible.

The posterior cardinal vein develops beneath the ectoderm as a loose plexus which eventually migrates medially and becomes closely associated with the Wolffian duct. Figure 8a is a SEM of a 20 somite embryo from which the ectoderm has been peeled off. The posterior tip of the Wolffian duct is marked by an arrow. Figure 9 is an immunofluorescent view of a similar area. At higher magnifications (Figure 8b), one observes the close association of PECs in this plexus as they approach the Wolffian duct. Eventually the PECs cluster around the Wolffian duct as can be seen in Figure 10 taken at a more anterior level of a 20 somite embryo.

Discussion

By utilizing both antibody labelling procedures and scanning electron microscopy, we have been able to observe and interpret the earliest stages of vascular development. The dorsal aortae arise from cells which segregate singly from the lateral mesoderm and aggregate to form longtiudinal cords at the lateral edges of somites and segmental plates. The posterior cardinal veins form from cells which segregate from the somatopleure confirming the suggestion of Hirakow and Hiruma (1981). They form from loose plexuses which move medially and become associated with the Wolffian ducts.

Vascular development can occur by two distinct morphogenetic processes: 1. *in situ* aggregation of groups of mesenchymal cells and 2. sprouting from pre-existing vessels (Wagner, 1980). We have shown that the posterior cardinal veins and dorsal aortae form by the first of these processes. The formation of the intersegmental arteries occurs by the second process, sprouting off the dorsal aortae. The sprouting form of angiogenesis has been studied more extensively as it is the mechanism by which tumors recruit a new vascular supply (Folkman, 1985). We are extending the descriptive analysis in this paper to other embryonic blood vessels and utilizing microsurgery and transplantation techniques to reveal the relative importance of these two processes in establishing embryonic vascular patterns.

Acknowledgements

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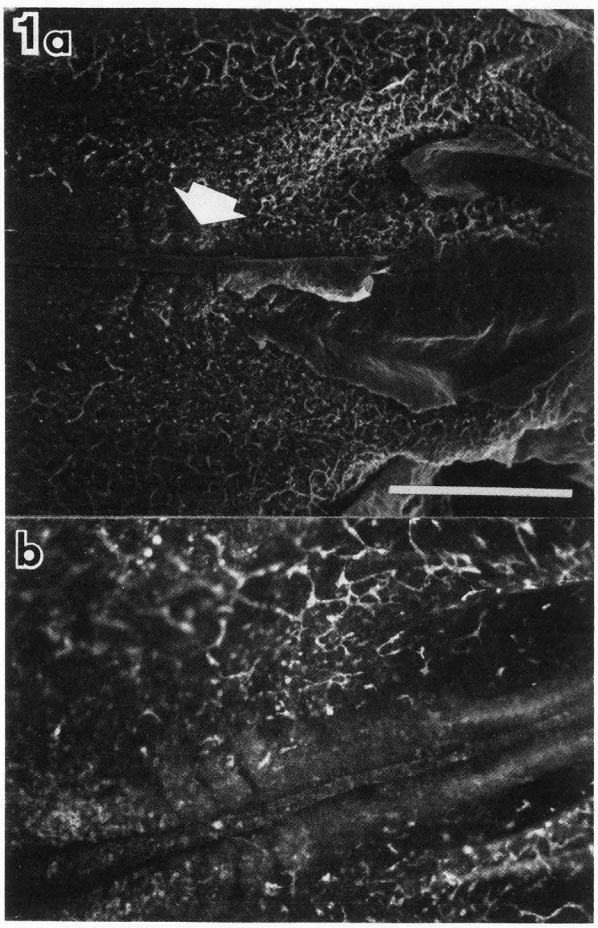

Figure 1a. 3 somite quail embryo with the endoderm peeled off. The first somite is indicated by an arrow. b. 3 somite quail embryo labelled as a whole mount with the monoclonal antibody, QH-1. Bar = 300μm.

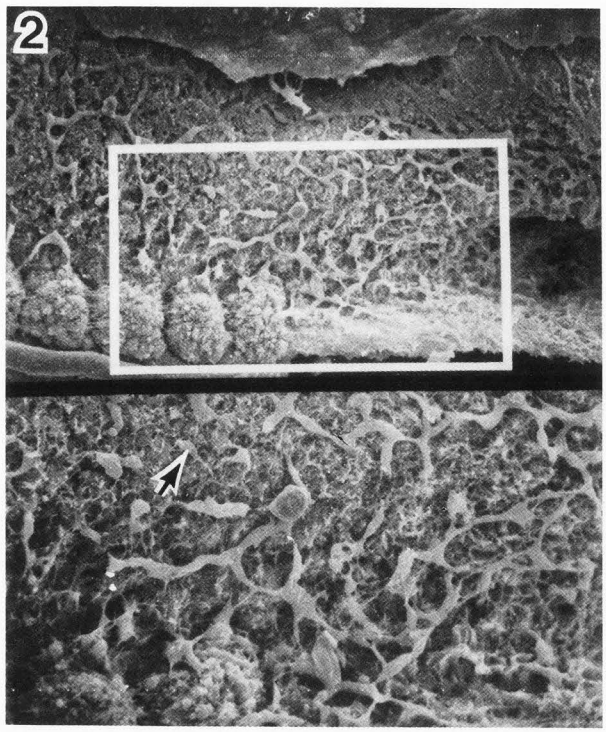

Figure 2. 5 somite quail embryo with endoderm removed. PECs adjacent to segmenting somites have now become clearly more numerous. An arrow indicates an individual PEC. Long side of white rectangle equals 300μm.

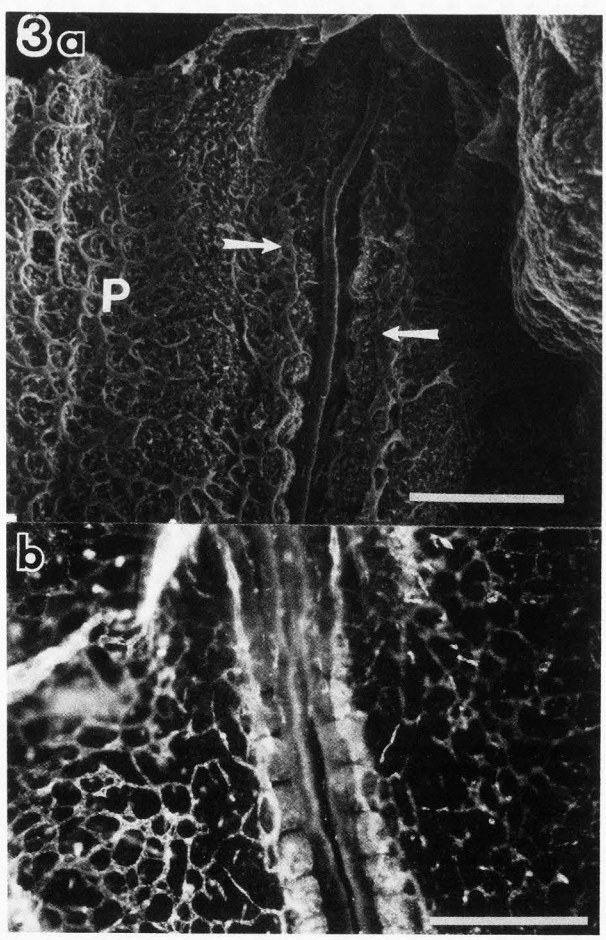

Figure 3a. As development proceeds, the cells of the dorsal aortae (arrows) form cords beneath the somites. Further laterally is a plexus (P) continuous with the extraembryonic yolk sac. 6 somite embryo. Bar = 200μm. b. A whole mount labelled with QH-1 shows clearly that these cells are PECs. Bar = 200μm.

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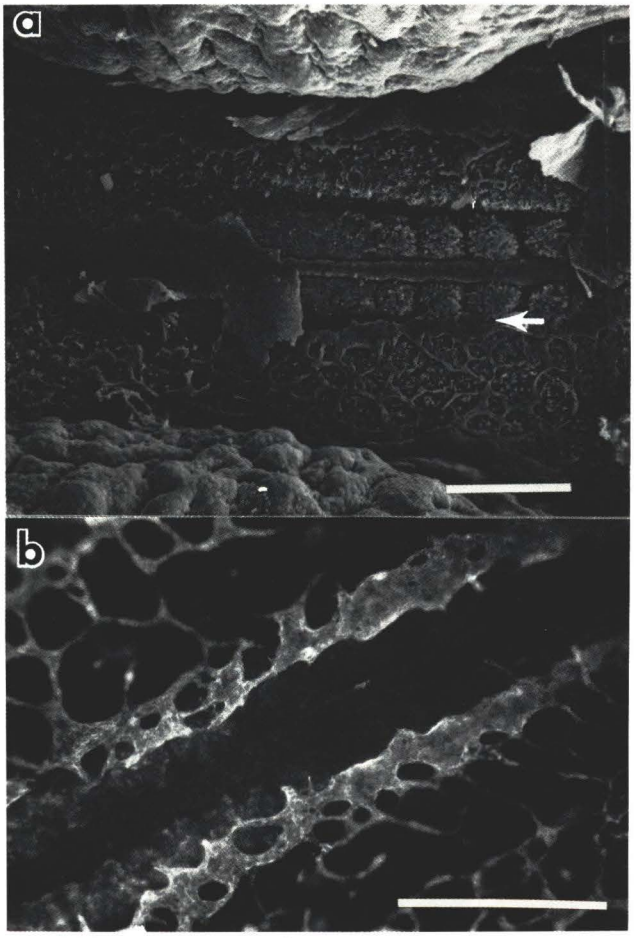


Figure 4a. In this photograph of a 7 somite embryo, the dorsal aorta on the left side (arrow) gives the appearance of a more continuous cord. The right dorsal aorta has been lifted off showing that it lies over a groove separating somites and segmental plate from adjacent lateral mesoderm. Bar = 200μm. b. This whole mount of a 7 somite embryo illustrates nicely the dorsal aortae, the gap which forms just lateral to them and the extensive plexus further laterally. Bar = 150μm.

Discussion with Reviewers

R. Kirakow: What is the reason to use the quail instead of the chick?

Authors: The QH-1 monoclonal antibody labels a cell surface antigen on quail endothelial cells. QH-1 does not label any chick cells. This

Figure 5. Here is a higher magnification SEM of a segment of dorsal aorta (arrow) in another 7 somite embryo. One observes an intimate association with the surfaces of the somites. Bar = 50μm.

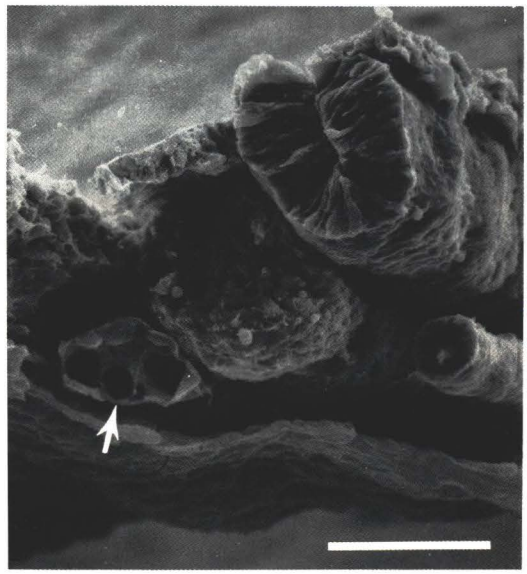


Figure 6. In cross-fractures as in this 7 somite embryo one observes multiple lumens in the dorsal aorta (arrow) separated by thin cellular processes. Bar = 50μm.

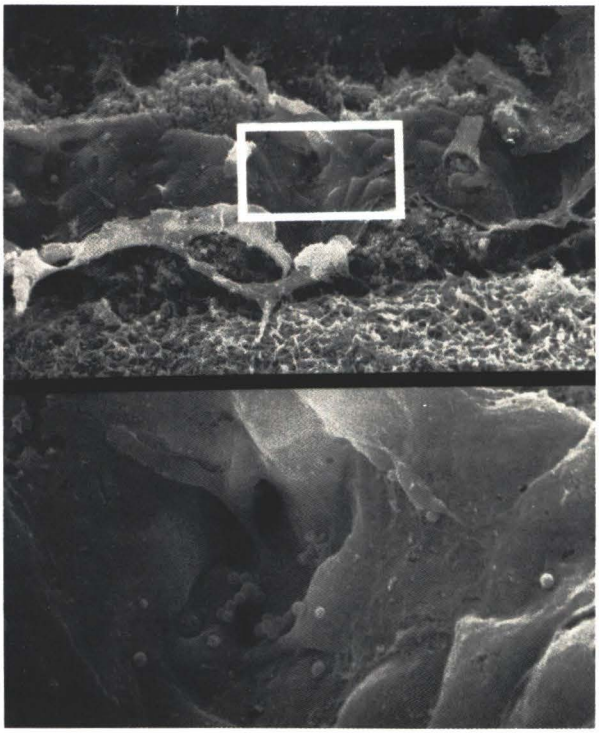


Figure 7. A 9 somite embryo's dorsal aorta which has been fractured open to reveal the luminal surface. The beginning of a branch, an intersomitic artery, is clearly visible. Long side of white rectangle = 50μm.

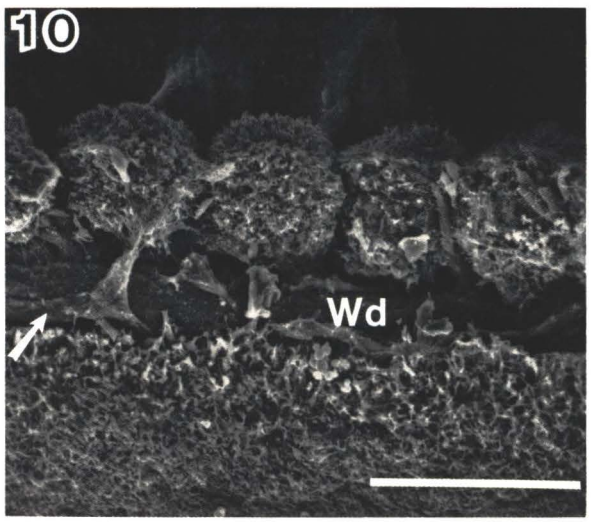


Figure 9. An immunofluorescent photograph of a 19 somite embryo. The central dark area is the neural tube. On either side can be seen the plexuses which will form the posterior cardinal veins. Bar = 100μm.

Figure 10. At more anterior levels of a 20 somite embryo, the PECs (arrow) are seen in close association with the Wolffian duct (Wd). Bar = 100μm.

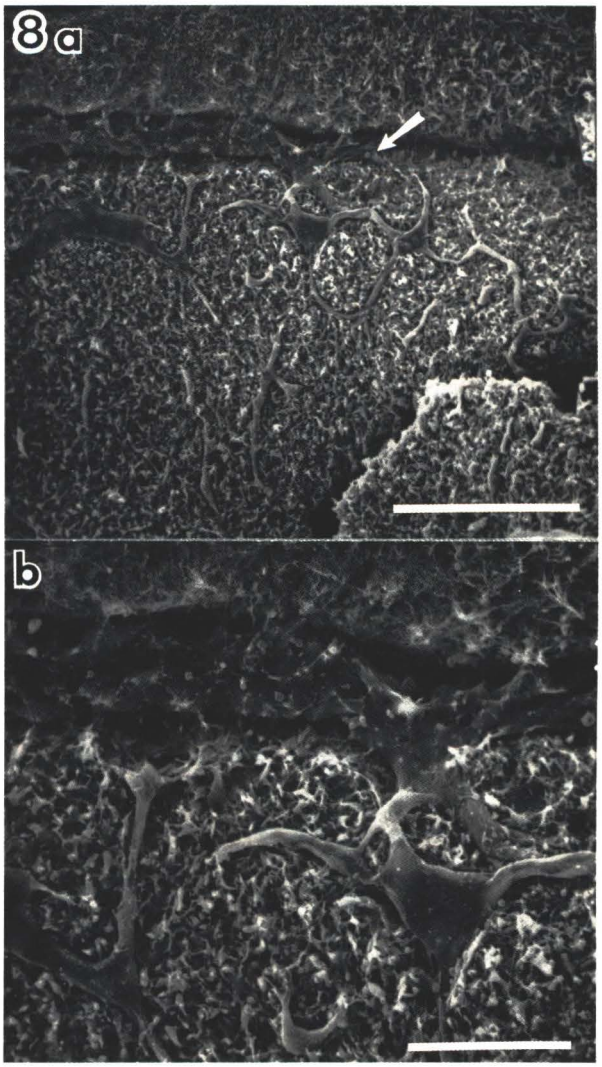
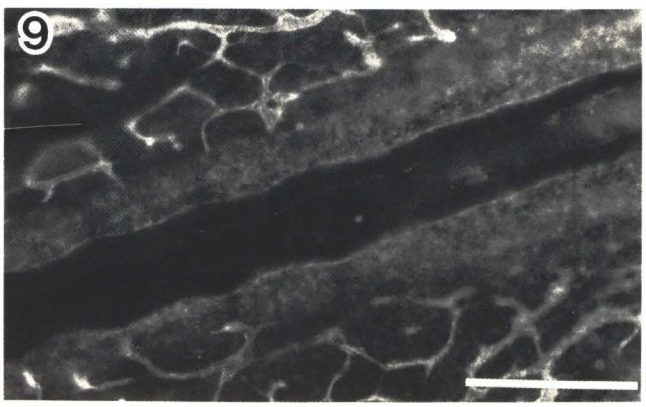


Figure 8a. The ectoderm has been peeled off this 20 somite embryo revealing the loose plexus of PECs which will form the posterior cardinal vein. The posterior tip of the Wolffian duct is indicated by an arrow. Bar = 150μm. b. A higher magnification SEM of an area of 8a showing the close association between PECs and the Wolffian duct. Bar = 50μm.



characteristic is of advantage for some quail-chick transplantation experiments which we are now pursuing.

E. Chernoff: The tissue was permeabilized for antibody staining, so cytoplasmic fluorescence is seen. Is it known whether the antigen is a cell surface component as well? Has the antigen for the QH-1 antibody been characterized? Is the antigen developmentally important? Does the antibody, or its Fab fragments, inhibit angiogenesis?
Authors: The embryos were permeabilized so that antibody could reach the mesoderm in whole mounts without peeling off the endoderm or ectoderm. The QH-1 antigen is a glycoprotein at the cell surface which is also shed into medium of cultured endothelial cells. It is as yet not very well characterized. We have not tried to inhibit angiogenesis with the antibody.

T. Pexieder: What kind of proof do you have for the segregation of prospective endothelial cells from the mesoderm?
Authors: Fate-mapping studies have indicated that blood vessel endothelium indeed derives from the mesoderm. We identify the cells we see segregating from the mesoderm as prospective endothelial cells by their binding of the monoclonal antibody QH-1, the lack of any other hemopoietic precursors which also stain with QH-1 in the embryo proper at these early stages of development, and their association with and morphogenesis into recognizable vessel rudiments.

E. Chernoff: There are morphological similarities between formation of the dorsal aortae and the very early stages of formation of the two primordial heart tubes. Do you think that similar processes are at work? Is there reactivity of the antibody with cardiac mesenchyme (splanchnic mesoderm) or early endocardium?
Authors: The processes of dorsal aorta and heart formation do appear similar. The formation of the primitive heart tube occurs in close proximity to the anterior intestinal portal. The closure of the anterior intestinal portal therefore has a physical influence on the morphogenesis of the heart tubes for which there is no equivalent in dorsal aorta morphogenesis. The antibody is reacting with prospective endothelial cells of the heart which are joining together to form the early endocardium.

P.B. Armstrong: Do you have any information on the processes responsible for the expansion of the population of primitive angioblasts during the period between their initial appearance and the establishment of the definitive endothelium of the major blood vessels? Does expansion involve continual recruitment from undifferentiated paraxial mesenchyme or is the extent of mitotic activity of committed angioblasts sufficient to produce the observed increase in cell number?
Authors: The mechanism of increase in cell number of the major vessel primordia is a central question in their morphogenesis. There is mitotic activity of PECs but it remains unclear what proportion of cells arises by mitosis. The answer to this question awaits the results of experimental intervention.

P.B. Armstrong: Do you have information on the processes responsible for the origin of angiogenic stem cells in regions craniad to the heart? Is there a similar process of differentiation *in situ* of the angioblasts of the major vessels or is the entire cranial vasculature formed by invasion of capillary sprouts from pre-existing vessels in neck and trunk regions?
Authors: Preliminary observations suggest that sprouting is the predominant form of angiogenesis in the head region. There is a craniad extension of the ventral aorta which branches and joins with the anterior limits of the dorsal aortae, forming the first aortic arch. The internal carotid arteries form as sprouts off of the first arch. However, we see some individual PECs over the developing brain before the formation of a complete plexus. Grafting and blocking experiments would clarify the origin of the angiogenic stem cells in this region.

R.N. Feinberg: All blood vessels do not persist throughout development. Will endothelial cells that undergo regression still label with the QH-1 antibody?
Authors: We are not sure. Most of our studies have involved early formation of the vasculature. The regression that you are referring to largely occurs later in development. However, there does appear to be some regression in early heart and dorsal aorta formation where degenerating strands do label.

P.B. Armstrong: Do you have any evidence to support the suggestion of Folkman and Haudenschild (1980, Nature 288, 551-6) that the vascular lumen originates as a series of intercellular vacuoles that fuse to form the ultimate lumen?
Authors: The reorganization of cords of endothelial cells into blood vessels with lumens seems to involve an assembly around an extracellular space. This has been studied by TEM in the case of the chick dorsal aorta by Hirakow and Hiruma (1983, Anat. Embryol. 166: 307-315) where they show an expansion of the intracellular space and enclosure of an extracellular space by attentuated PECs. They did not see large intercellular vacuoles.

P.B. Armstrong: Can the angiogenic stem cells be identified in cultures of axial mesoderm from embryos of the appropriate stages of development? Presumably important insights into developmental mechanisms could be realized if tissue culture model systems could be devised that expressed angiogenic processes similar to angiogenesis *in vivo*.
Authors: We have used the QH-1 antibody to identify endothelial cells in cultures of dissociated 7 day yolk sacs. We haven't tried earlier stages as yet, but we agree that this would be an important experimental approach to understanding vasculogenesis.