Vasculogenesis and Vessel Wall Differentiation in the Embryo

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Abstract

The study of vascular development in the embryo has concentrated on the role of endothelium and smooth muscle cells in both the formation of a vascular pattern and the establishment of the vascular wall. The endothelium, deriving from the mesoderm, passes through a number of stages, starting with precursors, and ensuing with strands, tubes, a plexus and ending in defined vessels as the dorsal aorta or cardinal vein. The endocardium of the heart deserves special attention in that it probably derives from two sets of precursors comprising a contribution of the heart-forming region as well as the medially located splanchnic mesoderm. Chicken-quail chimera studies show the plasticity of the vascular network. Cell movement and remodelling of the plexus are in the embryonic period ongoing processes. BrdU labelling shows that the dorsal aorta of a stage HH19-23 embryo does not harbour mitotic cells allowing for the conclusion that expansion of the aorta is possible through incorporation of endothelial precursors and cells from peripheral, smaller vessels where mitotic activity is considerably higher. The smooth muscle cells become apparent later in development. They derive from at least two sources, the splanchnic mesoderm and the neural crest. The latter is particularly active in the formation of the media of the pharyngeal arch arteries and their derivates. This is shown by retroviral-LacZ staining of the crest, double stained for muscle actin. The arch arteries appear to be formed by two differentiation waves of smooth muscle cells. It remains to be elucidated whether these two waves reflect cells of different origin. This can at present not be deducted from processes that lead to elastic and muscular artery formation or the future sites of arteriosclerosis in the vascular bed.

Key words: vasculogenesis, endothelial cells, vascular smooth muscle cells, mesoderm, neural crest.

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Development of the vascular system requires endothelial cells for lining of the lumen and ensuing smooth muscle cell differentiation for the formation of the media surrounding the endothelial tubes. The origin and subsequent differentiation of both cell types require further study as both have a major impact on the behaviour of vessels in normal and pathophysiological conditions. With regard to the origin of endothelial cells experimental data indicate the necessity for an endoderm-mesoderm interface for *in-situ* differentiation of endothelial cell precursors [21]. From this initial population endothelial cells are seen to proliferate and align to form endothelial tubes [6, 10, 11]. It remains to be investigated whether other embryonic mesodermal cell lines (derived from the heart-forming fields) can also contribute cells to the endothelial lining. This latter possibility should be considered seriously in view of the recently published results on endocardial cell origin [25].

There also is the question of endothelial cell mobility. Data from chicken-quail chimeras [5, 19, 22] indicate a marked capability of endothelial cells to move along the vascular wall. It is interesting to note that this movement seems to be predominantly counter current, that is towards the arterial pole of the heart [5, 19, 22]. In the present paper we will bring the data from our group concerning vessel formation in the thoracic region including the aortic sac, pharyngeal arch and pulmonary area.

A second aspect that will be covered concerns vascular smooth muscle cell origin. Data from literature indicate at least a dual origin, an initial set from the primitive streak mesoderm [8] and a second wave from the neural crest [24]. Recent data, using retroviral tracing of neural crest cells unequivocally support smooth muscle cell origin

from the neural crest [14, 18]. The specific function and the definitive role of neural crest cells in vessel wall differentiation, need further elucidation [24]. The last part of the paper will focus on aspects of smooth muscle cell differentiation and dedifferentiation that at least at present cannot be linked to a pattern of embryonic origin [14, 15].

Definitions

In describing vascular development in the embryo both from a patterning and differentiation point of view it is necessary to define the developing vessels and their contribuents. Vessel formation starts from isolated endothelial cells, so called endothelial precursors. The method to pick these cells up is by immunohistochemistry. For the quail there is the anti-endothelial antibody QH1 [6, 10] and in mouse embryos there has been described a PECAM1 antibody [2]. Recently for an endocardial cell line the antibody JB3 [25] has been described for the chick embryo. Dealing with QH1 we have to realize that it also stains the precursors from the hemopoetic cell line. These cells, however, have a different morphology when observed in tissue sections [22]. When the endothelial precursors start to form vessels a network is constructed that is made from tube parts and isolated cells. Depending on the position of these endothelial tubes we are inclined to refer to them as arteries and veins. For the understanding of the morphogenetic processes it may be too early to already characterize them as such. With subsequent differentiation of the media of the vessel wall the histologically acknowledged arteries, veins and capillaries develop. To overcome this problem we will describe in the embryo main vessels which on the basis of being connected to either the arterial pole or venous pole of the heart have already their classical annotation. Examples are the dorsal aorta, pharyngeal arch arteries, cardinal veins. These lumenized vessels can be studied by Indian ink injection [11]. The vessels are linked together by a vascular plexus that should not be referred to as a capillary plexus yet. As will be described in the results section this early endothelial network shows a high degree of plasticity allowing for extensive remodelling of the initial network to form more definitive vessels.

Arteries and veins will be defined definitively at a later stage of development after formation of a media comprising smooth muscle cells. Also in these later stages vessels are seen to disappear [11] but this process is considered to be different from the early remodelling of the endothelial lined tubes. In these older vessels (e.g. part of the sixth pharyngeal arch) both endothelial and smooth muscle cell disappearance is necessary.

The terminology of vasculogenesis and angiogenesis as described in the literature [23] is too rigid for use in embryonic development. In the embryo we have no problems in encompassing vessel formation by vasculogenesis in which locally differentiated endothelial cells line up to form endothelial tubes. Angiogenesis, defined as ingrowth of endothelial cells into an organ, e.g. in the neural tube [19] and aortic orifice [3, 22], seems to be more rare. A definition that is lacking refers to disintegration of endothelial tubes into isolated endothelial cells that are subsequently recruited for novel vessel formation. This process is described by us using the term "remodelling".

Material and Methods

For the early detection of endothelial precursors and initial endothelial tube and vessel formation, quail embryos were used from HH8 to HH34. These were studied with immunohistochemistry and ultrastructural techniques.

Endothelial cell mobility has been studied with the use of chicken-quail chimeras and RGD oligopeptides to inhibit migration. Additional information on endothelial cell behaviour was gained by using BrdU labelling experiments providing insight into spatial proliferation patterns of endothelial cell populations. Vascular smooth muscle origin and differentiation was studied in chicken embryos using immunohistochemistry and 3D reconstruction techniques. Retroviral labelling was used in chicken embryos to follow neural crest cells to their destiny in the outflow tract and the thoracic vessels.

Antibody staining was used throughout this study to discriminate between endothelium and smooth muscle cells or to study one of these cell types separately. Because a quail specific anti-endothelium antibody is available we performed many parts of our study in this species. The QH1 (αMB1) antibody (Hybridoma bank) was applied on sections or on whole embryo preparations as described by Poelmann [22]. The procedure for staining (smooth) muscle cells by HHF35, an anti-muscle actin antibody was described by DeRuiter [8].

Scanning Electronmicroscopy of quail embryos in HH5-HH13 was performed on glutaraldehyde-paraformaldehyde-fixed specimen. To expose the endocardium and endothelial layers the overlying yolk sac endoderm, splanchnic and somatic coelomic mesoderm or ectodermal epithelium were carefully removed. Furthermore, some embryos were sectioned frontally, sagittally or horizontally at chosen levels. The embryos were carefully rinsed in cacodylate buffer, postfixed in 1% OsO_4 and dehydrated in ethanol. Critical point drying over CO_2 was followed by sputter-coating with gold. After prior viewing in a Philips SEM 525M embryos were usually subjected to further preparation to remove debris or obstructing layers before coating again with gold and definitive study of the vasculature.

Chicken-quail chimeras were constructed as described earlier [5] to examine migration of vascular endothelial cells. In brief, white Leghorn and Japanese quail eggs were incubated to reach HH17-22. Parts of quail wings were grafted into chick host wings of corresponding stages. In a control series chicken wings were grafted into quail hosts. A number of chimeras were injected proximal to the site of implantation with a solution containing GRGDS at a concentration of 0.3 mg/µl. Two injections were placed separated by a 4 hour time span. After 1-5 days of reincu-

bation embryos were fixed, embedded in paraplast, serially sectioned and processed for immunohistochemistry. The quail endothelial cells were detected using the quail-specific anti-endothelial antibody MB1 or QH1, demonstrated by routine peroxidase staining.

BrdU experiments were performed in HH18-23 quail embryos to assess the ratio of dividing cells in various vessels. Seven embryos were marked at HH18 with 30 µl of a 2% w/v solution in saline during 19 hrs. At HH23 the embryos were harvested, fixed in 0.1% glutaraldehyde-2% paraformaldehyde in phosphate buffer for at least 2 hrs. After dehydration, specimen were embedded in Lowicryl K4M, hardened at -18°C under UV radiation (360 nm) for 2 days followed by room temperature posthardening. The thoracic parts of the embryos were serially cut in 1 µm sections. At least six sections 10 µm apart per embryo were pretreated with a proteinase K solution, followed by incubation in an anti-BrdU antibody (IU-4), 1:2000, followed by the second antibody RAM-PO and the third antibody SWAR-PO (Dakopatts 260 and 217). Peroxidase-activity was detected with a standard diaminobenzidine-peroxide procedure.

To avoid overestimation of BrdU-positive nuclei we first measured the diameter of endothelial nuclei in 100 cells, being 8.4 µm. Assuming that endothelial cells in the various parts of the vascular system do not differ in diameter, nor in their spatial distribution we felt safe to count the positive/negative BrdU ratio in every tenth section of 1 µm. At least six consecutive sections of five cranio-caudal levels were counted. Three vascular types were discerned: the dorsal aorta, both posterior cardinal veins and vascular plexus endothelium in the body wall. No left-right differences were encountered and craniocaudal variations between the level of the truncus arteriosus and the hindleg were not significant. Therefore, we collected all results as percentages per embryo.

Retroviral labelling in chick embryos was performed to determine the neural-crest derived smooth muscle cells of the pharyngeal arch region. The procedure as described by Noden [18] was essentially as follows. A replication-incompetent spleen necrosis virus-derived construct, harbouring a LacZ gene, was used to infect the neural crest of HH8-11 chicken embryos. Survival up tot HH35 allowed for the study of targets of NC-derived cells. The patterning of the NC-derived smooth muscle cells, fitting in the vascular walls of the pharyngeal arch arteries and their derivates were traced by double-staining for the presence of LacZ by standard galactosidase staining (blue) and with an anti-α-actin antibody HHF35, followed by conventional peroxidase staining (brown).

Results

Vasculogenesis

The first vascular network is seen in the splanchnic mesoderm facing the endoderm of the yolk sac in a stage HH6 (1 somite) quail embryo. This lateral vascular plexus extends towards the bilateral heart forming regions of the embryo. With medial fusion of the heart forming regions (± HH8) endothelial cells and precursors form a horseshoe-shaped plexus. This process of vasculogenesis in the early embryo takes place during foregut incorporation and cranial bending of the embryo. This results in a splanchnic vascular plexus that flanks the foregut. Ventrally there is the formation of large vessels (omphalomesenteric veins) that connect the yolk sac circulation to the developing heart tube. The endocardial lining of the heart tube is formed from endothelial precursors. There is no stage in which two separated endocardial tubes are found, giving rise to the incorrect stipulation of two fusing heart tubes. The ventral and dorsal mesocardium and the bilateral cardiac jelly formation give the erroneous impression that the endocardial lining of the heart is initially a double tube. The endocardial patterning is such that it connects to the well recognizable left and right omphalomesenteric veins, subsequently it becomes more plexuslike while at the arterial pole of the heart the formation of the first pharyngeal arch arteries (HH8) takes place. These connect to the part of the mesoderm dorsal from the gut, where the dorsal aortae are formed. Both SEM and immunohistochemical data point towards a network of endothelial precursors that already form a template for the subsequent vessels that will develop (Fig. 1). This is most obvious in the area flanking the foregut where the pharyngeal arch arteries are formed.

It is also clear that when taking into account both the lumenized vessels and the endothelial precursors that this comprises an extensive splanchnic plexus. Two important ongoing remodelling processes have not received much attention up till now. This concerns a strand of QH1 positive cells that is positioned ventral to the foregut and during development separates from the endocardium of the heart. It is pinched off by the surrounding mesoderm during myocardial tube formation and the disappearance of the dorsal mesocardium. This so called mid-pharyngeal endothelial strand (MPES) stays connected to the future sinus venosus and the aortic sac area (Fig. 2). The MPES is related to the already mentioned template of endothelial cells that surrounds the foregut. A second system concerns a bilateral vascular system in the body wall that, because of its position, has been termed the ventral pharyngeal vein. This vein and the anterior cardinal vein have many small connections to the developing splanchnic arteries (defined as the vessels that connect the aortic sac to the dorsal aortae). Both Indian ink injections and immunohistochemical studies confirm the patterning of the vascular plexus and its numerous "arterial-venous" connections. Remodelling in the early vascular plexus is an omnipresent feature. This is best seen following the vessel formation from cranial to caudal. The initial first and second arch arteries are broken down (Fig. 2) as well as the ventral pharyngeal vein and from the population of endothelial cells thus present recruitment for more definitive vascular network is seen.

We have followed the vascular formation and patterning up to HH30 at which the major vessels have been formed.

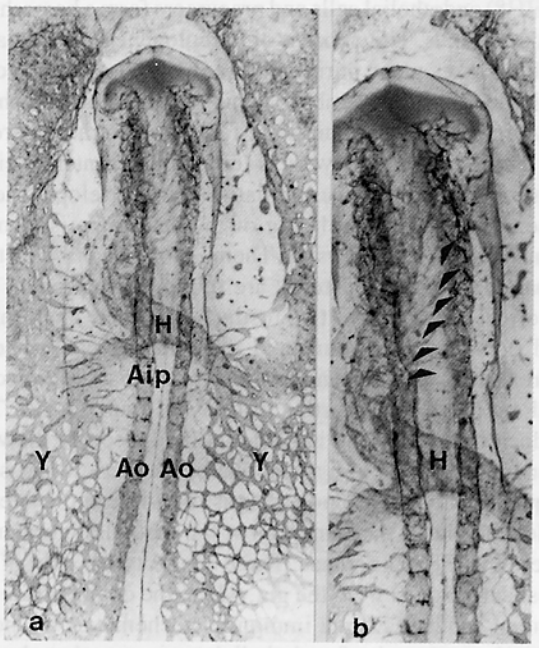

Figure 1. a: A quail embryo of stage 10 (10 somites) stained with QH1 for endothelial cells. A looping heart tube (H) is visible, somewhat cranial to the anterior intestinal portal (Aip). The foregut is flanked by endothelial precursors, a template for the ensuing pharyngeal arch arteries. The dorsal aorta (Ao) is connected to the yolk sac plexus (Y); b: Enlargement of Fig. 1a to show the endothelial precursors (arrowheads) flanking the foregut.

This leads to some interesting observations, that can be exemplified by describing the formation of the sixth pharyngeal arch artery and the pulmonary vasculature. First of all we have to realize that every arch artery develops from the vascular network that is present surrounding the gut. The classic description of two sprouts that simultaneously grow out [9] of both the aortic sac and dorsal aorta to fuse, thereby forming a connecting artery, is too simple as it does not account for the precursors and small tubes that are already present at this site (Fig. 2). Likewise the pulmonary arteries are formed in this way. In the lung primordium endothelial precursors are present from stage HH12-13 onwards. These cells become partly organized in tubes and can be followed through the splanchnic plexus up to the sixth arch (HH18). A similar continuity can be observed between the lung primordium and the MPES that is connected to the sinus venosus. This connection will develop into the pulmonary veins (Fig. 2). It is impossible to define these vessels as a future artery or vein as long as a media containing smooth muscle cells is lacking. From a hemodynamic point of view it is important to note that circulation can only be effected if the pulmonary artery primordium is lumenized along its complete length. The final lumen formation takes place in the area of its junction to the sixth arch explaining why the technique of Indian ink injection is inadequate to solve this part of the problem of pulmonary artery formation. After establishment of pulmonary artery continuity other vascular junctions to the lung primordium from e.g. the dorsal aorta disappear. Still this area is seen to harbour many isolated endothelial cells so that it is assumed that remodelling and renewed recruitment are mechanisms involved. Also somewhat later in time development of bronchial arteries occurs from the splanchnic plexus and the endothelial cells that are already present in the lung.

Endothelial cell mobility

To study this aspect quail-chicken chimeras were constructed by insertion of a part of a quail wing bud into a chicken wing bud. This resulted in a wing vascular plexus that was lined by a mixture of host chicken and grafted quail cells. The architecture of the plexus did not allow, at the stage studied, to differentiate between future arteries and veins. There was one peculiar phenomenon, however, in that grafted quail endothelial cells were found in a quadrant of the wall of the host aorta ipsilateral to the chimeric wing (Fig. 4). This implies that endothelial cells, by whatever mechanism, had moved antegradely along the arterial vessel wall. This experiment was confirmed in the reverse model in which a chicken graft was placed in a quail host showing a quadrant of QH1-negative, and therefore chicken endothelial cells in the quail dorsal aorta.

A second experiment to study whether endothelial cells migrated through cell-cell or cell-matrix interactions was a blocking experiment for migration. Extracellular matrix components were considered to facilitate migration and this adhesion was blocked by injection of an oligopeptide, containing a RGD sequence. In these experiments quail endothelial did not reach the aorta.

Endothelial cell proliferation

A set of experiments with BrdU labelling (Fig. 3) of embryos showed that the mitotic activity was higher in the peripheral plexus area (69%) and in those vessels that because of positional aspects were considered to become veins (52%). The dorsal aorta showed the lowest incidence of endothelial cell proliferation (33%). This was nearly completely due to a hot spot of high proliferative activity in the ventral aspect of the dorsal aorta at stage HH23. These cells were remarkable because of their cuboidal shape that differ from the squamous appearance of normal endothelial cells lining a lumen.

These data support the quail-chicken chimera results in that endothelial cells are most active in the still remodelling part of the vascular plexus and that the larger vessels increase in size by recruitment of endothelial cells from the periphery.

Smooth muscle cell origin

The first indication of smooth muscle cell differentiation as detected by actin positivity was seen in the HH16 chick

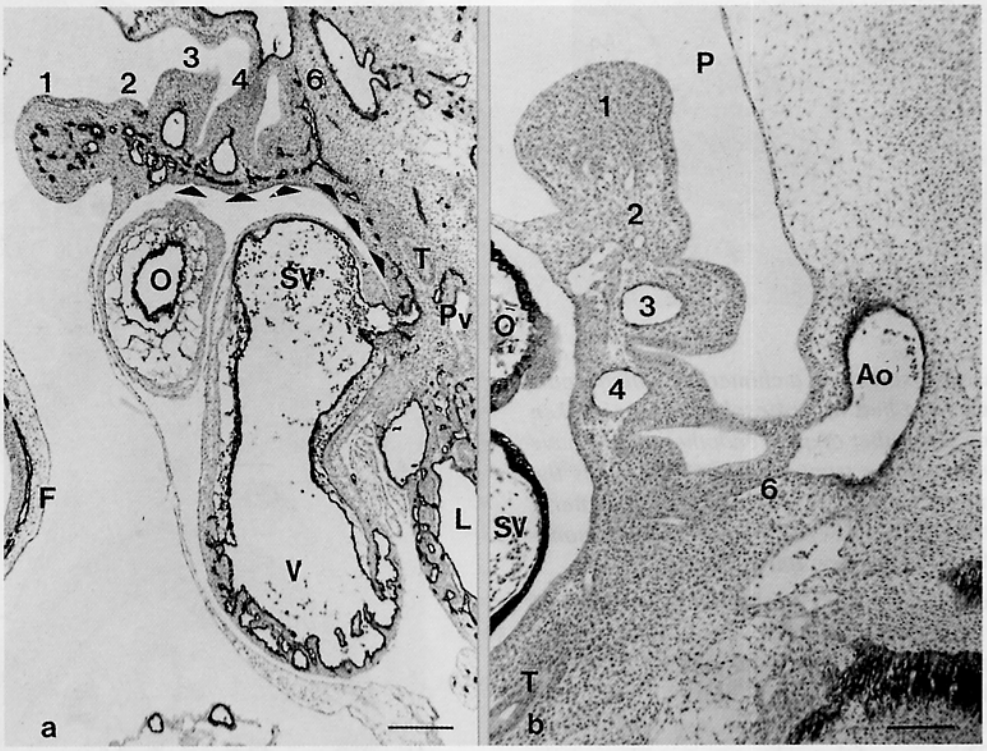

Figure 2. a: An almost median section through a quail embryo of HH19 incubated with QH1 to show the endothelial cells. The pharyngeal arch arteries 3 and 4 are evident, whereas 1 and 2 have already disintegrated into a plexus. At the site of the developing sixth a small plexus can be discerned. Note that this plexus joins with the midpharyngeal endothelial strand (arrowheads) that continues alongside the trachea (T) to hook up with the sinus venosus (SV), and the first sign of the pulmonary vein (Pv). Bar = 60 μm. Ao = aorta, F = forebrain, L = liver, O = outflow tract, V = ventricle. b: Enlargement of the pharyngeal arch region of a section adjacent to that depicted in Fig. 2a, stained with HHF35 for α-muscle actin. Note the subendothelial presence of actin in the aorta (Ao). Also arch artery 3 and 4 contain minute amounts of actin. O = outflow tract, T = trachea, P = pharynx, SV = sinus venosus, Bar = 100 μm.

embryos. This subendothelial layer of actin positive cells spreads along the vessel wall in a caudal to cranial sequence, starting at the dorsal aorta and reaching the arterial orifice level at the outflow tract of the heart (Fig. 2b). These smooth muscle cells either develop *in-situ* in a subendothelial position or migrate through the mesoderm. There is no evidence for the latter supposition as outside the condensed mesenchyme of the media of the vessel wall no actin positive cells were seen. It is obvious that not all cells that form the compact layer of the future vessel wall media show actin positivity. The timing of this differentiation implies that the surrounding mesoderm itself is the source for this first echelon of vascular smooth muscle cells.

From HH19 onwards neural crest cells arrive in the area of the aortic sac. This has been described in chicken-quail chimera experiments. We have confirmed this with our tracing of neural crest cells after retroviral labelling (Fig.

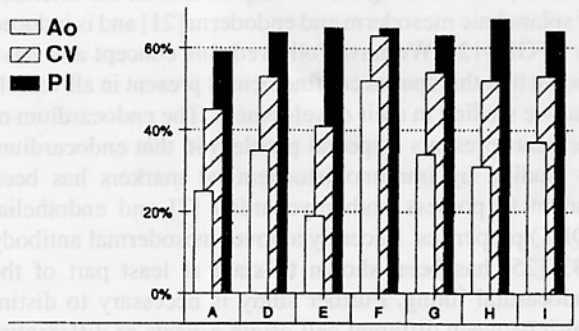

Figure 3. Percentages of BrdU-positive nuclei in endothelial cells of 7 quail embryos (A-I). The embryos were labelled at HH19 and analysed at HH23. Ao = dorsal aorta, CV = posterior cardinal veins, PL = plexus in body wall. The aorta was always lowest, whereas the plexus was always high.

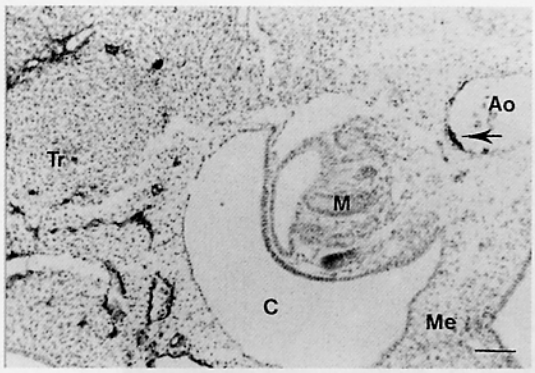

Figure 4. Transverse section of a chimera in which a part of a quail wing bud was transplanted to a chicken host wing. A number of quail endothelial cells have migrated from the transplantate (T) towards the dorsal aorta (Ao) and have settled in the ipsilateral face of the aorta (arrow). C = coelom, M = mesonephros, Me = mesentery. Bar = 50 μm.

5a,b). Blue LacZ staining cells are found in the media of the aortic arch arteries and the great vessels at the arterial pole of the heart (ascending aorta and pulmonary trunk). Immunohistochemical staining with anti-α-actin shows that part of the retroviral marked neural crest cells are also actin positive. This is proof that at least transiently an actin positive population of crest-derived smooth muscle cells is present in the vessel wall.

Discussion

Vasculogenesis in the embryo has turned out to be a complicated process in which *in-situ* differentiation [21], migration [5, 19] and patterning and remodelling [11] play an important role.

The origin of the endothelial cell is still not perfectly clear. It is shown that endothelial cells, characterized by their expression of QH1, develop in-situ on the interface of splanchnic mesoderm and endoderm [21] and is induced by bFGF [12]. We have followed this concept and have shown that this spatial confinement is present in all vessels that we studied in their development. The endocardium of the heart presents a special problem in that endocardium as studied by immunohistochemical markers has been shown to possess both myocardial [7] and endothelial (QH1) properties. Recently a novel mesodermal antibody JB3 [25] has been shown to stain at least part of the endocardial lining. Further study is necessary to distinguish between different cell origin aspects or differentiation capacities of one cell line. The mobility of endothelial cells in the embryo seems to reflect active migration [19]. However, other factors might be equally important. It is clear from the study of serial sections and whole mounts with immunohistochemistry (QH1) that an endothelial precursor population is active in endothelial tube forma-

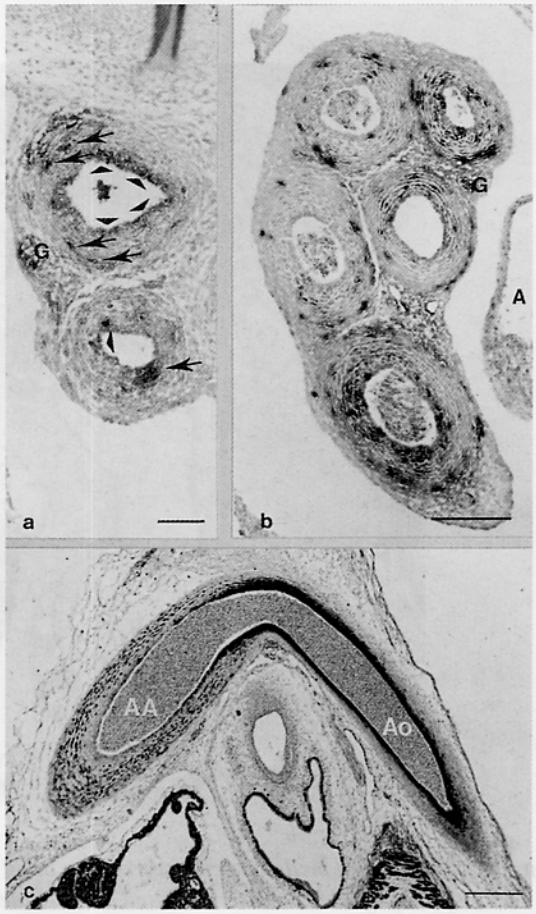

Fig. 5. a and b: Two levels of the arterial pole of a chicken embryo. Fig. 4a is in the neck region, while 4b is close to the heart. The neural crest of this embryo has been retrovirally marked in stage HH10 and the embryo survived until stage HH30. The sections are double-stained for α-actin and LacZ activity. The actin staining is diffuse and adjacent to the lumen in Fig. 4a (arrowheads), but absent in Fig. 4b. Retrovirally marked cells (arrows) are present throughout the media. Note that in Fig. 4b the SMC are arranged in lamellae, while in 4a this lamellar arrangement is not evident. A = atrium, G = vagal ganglion. Bar = 100 μm. c: is a sagittal section, HHF 35-stained of a normal HH32 embryo, showing the aortic arch. Note the lamellar pattern in the ascending aorta (AA) with alternating actin positive and negative cells. The descending aorta (Ao) shows a diffuse actin staining, which is strongest at the luminal side. Bar = 50 μm.

tion. This implies that cell-cell and cell-matrix interactions are important factors in this remodelling process. Experimental embryonic data show that interference e.g. with extracellular matrix receptor by blocking with oligopeptides leads to alterations in vascular development [5]. More serious vascular inhibition leading to embryonic cell death is to be expected in transgenic animals that have a knock-out of their extra cellular matrix receptor as shown by e.g. Baldwin for VCAM [1].

The mechanism by which endothelial cells tend to move counter current along the arterial wall is still not understood. This mechanism has been reported by several groups [5, 19, 22]. A hypothesis we would like to propose is that very little active migration is necessary and that passive incorporation of endothelial cells into a growing artery might explain the rapid disappearance of endothelial lined tubes in remodelling areas [11]. To gain more insight in differences in endothelial cell behaviour in the vascular plexus and larger arteries we performed BrdU labelling experiments. These clearly point towards a higher mitotic activity in the plexus and along the veins. This supports the idea that arteries grow at expense of the peripheral network as shown also by the chimerization experiments using wing buds.

It is still questionable in which way vascularization of organs in the embryo is established. As indicated our studies show at least for the lung an initial *in-situ* population of endothelial cells. In the vascularization of the heart effected by the development of the coronary vasculature, quail-chicken chimeras have given more insight. In these embryos a piece of quail epicardial tissue containing endothelial precursors was placed in the cavity of the yet epicardium-free hearts. The quail endothelial cells were seen to move subepicardially and invade the myocardium and eventually the aortic orifice of the heart [3, 22]. The presence of great numbers of quail endothelial cells in these chimeras show that migration and proliferation both are important factors in the formation of the coronary vasculature.

The first sign of vascular media formation is considered to be the appearance of actin positivity directly subendothelially. In both rat [9] and chick (present study) actin is first expressed in arteries and only later on in the veins. There is also the tendency for actin to be expressed in relatively stable parts of the vessel wall that will not remodel extensively. These actin positive cells are of mesodermal origin. From HH19 onwards a second wave of mesectodermal (neural crest) derived smooth muscle cells invade the pharyngeal arch arteries and the wall of the aortic sac. At this time there is a peculiar phenomenon which is especially obvious in the aortic sac. The wall of the aortic sac (ascending aorta and pulmonary trunk) becomes layered and looses actin expression. The actin is reexpressed in a later stage in a reverse pattern across the vessel wall. The possible role of the neural crest cells still remains to be elucidated. The waves of protein expressions across the vessel wall have been observed for various contractile and cytoskeletal proteins as well as extra cellular matrix components [4, 24].

Our retroviral experiments do not indicate that neural crest cells can be the explanation for media differentiation of the vessel wall into either a muscular or elastic vessel wall type. The ductus arteriosus for example does receive a neural crest cell contribution but it develops into a muscular artery wedged between elastic arteries. The elastic dorsal aorta is not seen to receive neural crest derived smooth muscle cells.

Likewise, no indication is found, that later on during intimal thickening formation in arteriosclerosis a neural crest cell derived population plays a separate role. Predelection sites for arteriosclerosis do not coincide with those vessels in the body that either do or do not contain neural crest cells. The question whether the smooth muscle cell phenotypes, that have been detected by morphological features [16, 17, 20] are related to an embryonic cell lineage remains to be solved.

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