Development of the Cardiac Coronary Vascular Endothelium, Studied With Antiendothelial Antibodies, in Chicken-Quail Chimeras

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The endothelium of the coronary vascular system has been described in the literature as originating from different sources, varying from aortic endothelium for the main coronary stems, endocardium for the intramyocardial network, and sinus venosus lining for the venous part of the coronary system. Using an antibody against quail endothelial cells (α-MB1), we investigated the development of the coronary vascular system in the quail (Hamburger and Hamilton stages 15 to 35) and in a series of 36 quail-chicken chimeras. In the chimeras, pieces of quail epicardial primordium and/or liver tissue were transplanted into the pericardial cavity of a chicken host. The results showed that the coronary vascular endothelial distribution closely followed the formation of the epicardial covering of the heart. However, pure epicardial primordium transplants did not lead to endothelial cell formation, whereas a liver graft with or without an epicardial contribution did have this capacity. The first endothelial cells were seen to reach the heart at the sinus venosus region, subsequently spreading through the inner curvature to the atrioventricular sulcus and the outflow tract and, last of all, over the ventricular surfaces. At these sites, the precursor cells and small vessels were seen to invade the sinus venosus wall, the ventricular and atrial myocardium, and the mesenchymal border of the aortic orifice. Connections with the endocardium of the heart tube were only observed in the right ventricular outflow region. Initially, the connections with the aortic endothelium were multiple, but later in development only two of these connections persisted to form the proximal part of the two main coronary arteries. Connections to the pulmonary orifice were never observed. Our transplantation data showed that the entire coronary endothelial vasculature originated from an extracardiac source. Moreover, using the developing subepicardial layer as a matrix, we showed that the endothelial cells reached the heart from the liver region. Ingrowth into the various cardiac segments was also observed. Implications for the relation to specific congenital cardiac malformations are discussed. (Circulation Research 1993;73:559-568)

KEY WORDS • epicardium • congenital heart malformations • fistulous connections • heart development • vessel development

H uman hearts are subject to various coronary vascular abnormalities that may lead to a number of diagnostic and surgical problems. In pulmonary atresia without a ventricular septal defect, coronary vascular abnormalities vary, for example, from numerous fine intramyocardial sinusoids to large ventriculoarterial connections (so-called fistulas).1,2 Other malformations are characterized by an abnormal origin of a coronary artery from the pulmonary orifice3 or an abnormal origin combined with an abnormal proximal course.4,5

Data concerning coronary vascular development are insufficient to understand the basic underlying mechanisms for these abnormalities. Are these the results of persisting normal connections, an initial abnormal anlage, or secondary events developing late in gestation?

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Current knowledge regarding coronary vessel development shows that the embryonic heart is already pumping before the wall is vascularized and perfused by its own nutritive circulation. The human embryonic heart beats at 3 weeks of gestation, whereas coronary arterial orifices in the aorta are present at 37 days of gestation.6 This difference is also seen in animals. The rat heart pumps at day 9, and coronary arterial connections are seen at day 17.7 The chicken heart contracts at day 2, with coronary arterial connections at day 7 or 8.7 The coronary vascular network has been described as developing in close association with the epicardial covering of the heart tube. The epicardium arises as an outgrowth of the celomic wall6 initially covering the liver region. In the chick embryo, the epicardium is seen to cover the heart between days 3 and 5.8-12 Thereafter, a vascular network develops in the subepicardial layer positioned between the myocardial heart tube and the mesothelial layer of the epicardium. As mentioned above, this network eventually contacts the semilunar sinuses of the aorta in clearly defined areas to form the coronary arterial orifices.13 Connection of the network with the sinus venosus has also been found to occur
after formation of the epicardium, however, without indicating an exact stage of development. The intramyocardial part of the vascular system poses an additional problem as to the origin of endothelial cells lining these vessels. For this intramyocardial network, endocardial cells as well as subepicardial precursors have been mentioned as a source. In the present study, the origin of the coronary endothelial cells was investigated in normal quail embryos as well as in chicken-quail chimeras. Chimeras were constructed by inserting pieces of the liver underlying the epicardial organ into the pericardial cavity of the chick embryo. The endothelial cells of the quail can be recognized with immunohistochemistry, using quail-specific antiendothelial antibodies. In this way, we expected to resolve questions regarding the origin of the coronary vascular endothelium both for the subepicardial and intramyocardial network.

In addition, we present data that might explain variations in coronary vascular patterns seen in both animal and human hearts that are regarded as pathological when leading to clinical symptoms.

Materials and Methods

White Leghorn chick (Gallus domesticus) and Japanese quail (Coturnix coturnix japonica) embryos were used in this study. Both the chicken and quail embryos were staged according to the criteria of Hamburger and Hamilton.

Normal Quail Embryos

For the description of normal vascular development, a series of normal quail embryos from Hamburger and Hamilton stages 15 to 35 (HH15 to HH35, 3 to 8 days of development).
incubation) was used. The embryos were processed as described below in “Immunohistochemistry.”

Chimeras

For the transplantation experiments, HH11 and HH15 to HH24 quail embryos were used as donors. The chick host embryos used were at stages HH7 and HH14 to HH20. The survival time after the implantation of the quail tissue varied from 3 days up until hatching (Table). In an initial set of experiments, care was taken to transplant selectively a nonvascularized epicardial primordium. In these embryos, we could not trace any quail endothelial cells in the host by our immunohistochemical techniques. Thereafter, the experiments were continued by implanting pieces of tissue predominantly containing liver. As the liver was cut down into several pieces, these varied from solely liver to liver including the covering epicardium. Only one set of experiments was different, represented by embryo 0 (Table), in which a piece of body wall including the sinus venosus was not transplanted into the pericardial cavity but into the body wall of a young chick host, close to the heart anlange.

The following transplantation technique was used. First, the ventral body wall of the quail embryo was opened to expose the heart. The heart tube was carefully removed, exposing the dorsal celomic wall and the epicardial primordium covering the liver region. The liver tissue to be transplanted was excised by fine tungsten needles. The transplant was transferred to the pericardial cavity of a chicken embryo (HH14 to HH18) through the naturally existing hiatus in the body wall (Fig 1) until this hiatus closed at stage HH18 together with the completion of the amnion. At stages HH18 to HH20, the transplant was brought into the pericardial cavity through a small cut in the body wall in the same region.

The location of the transplant at the time of the experiment, either by gentle manipulation or by the force exerted by the contracting heart tube, was either in the inner curvature of the loop of the heart or close to the sinus venosus region. After death, the successful implants proved to be either located near the sinus venosus area, the atrioventricular sulcus, or the outflow region (Table). The decision whether a graft was really of quail donor origin was based on the typical quail nucleolar staining. The embryos were processed for immunohistochemistry to detect specifically the quail endothelial cells.

Immunohistochemistry

The normal quail embryos and the chimeras were fixed in a mixture of 2% acetic acid in ethanol at 4°C for 24 to 27 hours. After parafin embedding, the embryos were serially sectioned and transferred to albumin/glycerine–coated objective slides. The 5-μm sections were incubated to detect the presence of the quail-specific MB1 protein, an antigen present on the surface of the quail endothelial cells, leukocytes, and their precursors. α-MB1 (kindly provided by M. Coltey, No- gent-sur-Marne, France) does not stain chicken cells. The following procedure was followed for the incubation. The slides were incubated with the α-MB1 antibody diluted in phosphate-buffered saline (PBS), to which had been added 0.05% Tween 20 and 0.1% bovine serum albumin at 20°C for 24 hours. After thorough rinsing in PBS and Tween 20, the slides were incubated with the second antibody, peroxidase-conju-gated rabbit anti-mouse immunoglobulin (Dakopatts P 260), followed again by rinsing in PBS and Tween 20. The location of peroxidase activity was determined with diaminobenzidine (Sigma Chemical Co, St Louis, Mo)
and H₂O₂. The sections were briefly counterstained with hematoxylin and covered with Entellan.

Definitions by Antibody Staining

The antibody used, α-MB1 specifically stains quail endothelial cells, leukocytes, and their precursors. In this study "endothelial cell" is used whenever a cell stains positive and lines a vascular lumen. "Precursor" is used when a positive cell does not line a lumen. These cells appear individually or in small clusters. Positive cells in the lumen of major blood vessels are considered to be leukocytes. From the earliest stages studied, vessels are present, and precursors are found in numerous places within the mesenchyme of the embryo. In the stages described, the vascular network mainly consists of endothelium-lined tubes before the formation of vessel wall media. This implies that a differentiation between arteries, veins, and capillaries is not yet possible. An exception can be made for the main coronary arterial stems, which are associated with a compact layer of surrounding cells that will develop into media. These vessels can be classified as arteries because of their specific connections to the aorta. The same can be posed for connections to the sinus venosus that will develop into veins.

In the specimens studied, the inner lining of the heart, the endocardium, also stains positive. This provided us with the technical difficulty of distinguishing between the contribution of the epicardial network and the endocardium or inner lining of, for example, the aorta and sinus venosus to the coronary vascular network. The experimental approach of constructing chimeras has been designed to make this distinction possible.

Results

Normal Development in Quail Embryos

Epicardial formation. The celomic surface of the primitive heart tube was not yet lined by epicardium in an HH14 to HH15 embryo. In the region of the developing liver, the first indication of the epicardial primordium was seen at this stage. At HH16 to HH17, this tissue consisted of mesothelium-lined protrusions that were filled with loose mesenchyme containing extracellular matrix components. This epicardial primordium, which was seen to increase in size in the following stages (HH18 to HH24), eventually covered the myocardium of the heart tube. The first contact with the heart was made at the sinus venosus region. The epicardium then spread initially into the atrioventricular sulcus, reaching the inner curvature of the heart tube. From there it reached over the outflow tract and the ventricular and atrial surfaces (Fig 2, a). The thus-formed epicardium consisted of a continuous one-cell-thick outer lining and a loosely structured subepicardial mesenchyme. This latter mesenchyme was more obvious in the sulci of the heart and was often hardly visible over the myocardium of the ventricles and atria. At HH19, the first endothelial precursors were detected in the mesenchyme of epicardium near the sinus venosus (Fig 3, a and b). Also, small vessels were seen connecting the liver sinusoids with the lumen of the sinus venosus (Fig 3, c).

The liver and sinus venosus region. At HH16, a relatively small liver primordium was seen close to the foregut. In the surrounding mesenchyme, some vessels and precursors were detected. By HH22, the liver primordium was well vascularized by numerous intraparenchymal sinusoids. These sinusoids drained from HH17 onward into the sinus venosus, establishing a first vascular contact with the heart tube. The impression
exists that these vessels grow from the liver region into the sinus venosus and not the reverse. The chimeric studies (see below) supported this view.

The atrioventricular region and ventricular myocardium. From H15 to HH24, MB1-positive cells were solely present in the endocardial lining in these areas. The first precursors in the subepicardial tissue of the atrioventricular region were detected around HH24. These reached this area from the already described sinus venosus region by way of the dorsal mesocardium. At HH25, the first endothelium-lined vessels were observed in the atrioventricular sulcus, developing into a dense network (Fig 4, a). From HH26 onward, the precursors were present not only in the subepicardial layer but also in the compact outer layer of the myocardium. Here, a fine network of vessels became apparent (Fig 4, a and b). In sections, this network appeared as regular profiles occasionally connected to the subepicardially located vessels. The impression exists that they do not directly contact the endocardial lining, except in the outflow of the right ventricle (see below). We were again confronted with the problem that we could not distinguish between endocardially and subepicardially derived endothelial cells.

Arterial orifice level connections. Between HH28 and HH35, the vascular system located in the atrioventricular sulcus of the inner curvature of the heart tube was seen to expand toward the truncus arteriosus, both as endothelium-lined vessels and as precursors (Fig 5, a and b). In the loose mesenchyme around the aortic orifice of 6-day-old embryos (HH33), a network of vessels and precursors was seen, forming a periarterial cuff that also ran between the aortic and pulmonary orifice. The left and right sides of this cuff were not located at the same mesenchyme-myocardial interface. The vascular cuff, close to the mesenchymal-myocardial border of the aortic orifice, showed a more myocardium-related position over the outflow tract of the right ventricle, surrounding the pulmonary orifice. These vessels extended into the right ventricular outflow tract myocardium. Contact between the endocardium and the vessels of the periarterial cuff could not be excluded.

In HH33 embryos, the cuff was connected to the three aortic semilunar valve sinuses. In the aortic wall of each sinus, approximately three or four endothelial cell strands were seen, occasionally containing a discernible lumen. These endothelial strands (Fig 5, a) were found...
to enter the mesenchymal aortic wall close to the muscular tissue of the outflow tract of the left ventricle. By HH35, two of these connections deserved special attention because they were already further differentiated into the most proximal part of the left and right coronary artery. Surrounded by media consisting of smooth muscle cells, these connections presented with a wide lumen that could even be noticed at low magnification. The orifices were invariably found in the two so-called facing sinuses of the aorta that were adjacent to the pulmonary orifice. The non-facing valvar sinus, close to the atrial wall, was only reached by endothelial strands lacking both a medial condensation and a lumen. The main arteries could be traced over some distance in the periarterial cuff to the site where they merged with the vascular network in the subepicardial mesenchyme over the sulcus and ventricles of the heart. At the pulmonary orifice level, no ingrowth was seen. As described above, around this orifice, especially in the area of the non-facing sinus, the vasculature was positioned subepicardially compared with the myocardium.

**Chicken-Quail Chimeras**

**General.** After survival times up to hatching (Table, embryo 10), the quail grafts developed without losing their original tissue specificity. As described in “Materials and Methods” the exclusive grafting of epicardial primordium did not lead to endothelial cell development. These experiments were not continued. A small set of experiments represented by embryo 0 in the Table allowed us to study the initial colonization of the epicardial primordium by endothelial precursors. This very young chimera showed that endothelial precursors migrated from the dorsal body wall into the epicardial protrusions to reach the pericardial surface of the heart tube, using the native host epicardium as a pathway.

The positioning of the liver/epicardium graft at the time of surgery already indicated that the graft was easily caught in the sinus venosus region near the liver or in the atrioventricular sulcus at the inner curvature of the heart. In the Table, we indicated where the liver transplants were found at the time of death. There were two possibilities, depending on which tissue was invaded by the liver. If the quail graft was positioned near or in the liver of the host, the host liver became truly chimeric. The quail liver cells were detected by their characteristic nucleoli. If the transplant was attached to the atrioventricular sulcus, for example, it was encapsulated by the host epicardium but kept its quail liver characteristics. The quail endothelial cells, however, were found at variable distances from the transplant. The vessel network that harbored the quail cells was identical to the pattern seen in the normal quail embryos, which made us confident that the transplanted endothelial cells followed the normal patterning of the vasculature in the chick. The predominant attachment sites are schematically depicted in Fig 2, b. Some transplants showed only ingrowth into noncardiac areas. Location in the sinus venosus region could lead to selective ingrowth in liver (embryos 9, 12, 13, 27, 29, and 34) or body wall (embryos 1, 2, and 32). Location at the periphery of the outflow tract showed an ingrowth in the body wall (embryos 8, 18, and 33) or no ingrowth at all (embryo 22). The description of our results focused on those transplants that contributed endothelial cells to the heart. For clarity, not every chimera but only the distribution pattern of the cells at their destination in the various segments of the heart will be described.

**Sinus venosus.** The grafts that were positioned near the sinus venosus or even in the liver itself were seen to contribute quail endothelial cells to the lining of the sinus venosus (Fig 6, a). Also, the subepicardial and myocardial walls of the atrium were, in some cases, invaded. Because there is a gradual transition from sinus venosus lining to atrial endocardial lining, it could not be established whether the atrial endocardium was also invaded. It was also clear that the direction of endothelial cell ingrowth tended to be downstream (eg, from the liver toward the sinus venosus) and not upstream (eg, from a transplant in the atrioventricular sulcus toward the liver sinusoids).

**Atrioventricular sulcus.** An extensive network of vessels and graft-derived precursor cells was seen in the sulcus; this network extended in several directions in the subepicardial layer. From there, endothelial cells were seen to invade the myocardium. In some embryos, a pathway was observed between the myocardium and the atrioventricular cushion tissue, the site of atrioventricular valve formation (Fig 6, b).

**Atrioventricular myocardium.** Dependent on the site of implantation and the survival time, a more or less extensive intramyocardial network was seen. One embryo that survived until hatching showed parts of the myocardium that were colonized by a very complete quail endothelium–lined network (Fig 7, a). From this experiment, it became evident that almost the complete wall from the epicardium to endocardium was colonized by endothelial cells derived from the graft. Their origin could be traced to the subepicardial layer. A meticulous search for connections of this network with the ventricular lumen proved to be negative (Fig 7, b and c), except for some connections in the outflow tract of the right ventricle, as was already described for the normal quail embryo. We also could not find a mixing of the endocardial lining and the quail endothelium. These results indicated that in these chimeras there is no contribution of the endocardium to the lining of the intramyocardial vascular network.

**Arterial orifice level.** A large number of quail-derived endothelial cells were found in the periarterial cuff (Fig 8, a through d). In general, the pattern is similar to that described for the normal quail embryo. An ingrowth of numerous endothelial strands in the three semilunar valve sinuses was seen; it was also present in the so-called noncoronary sinus (Fig 8, a). Only two large coronary vessels were seen to possess a muscular media (Fig 8, c) and a lumen contact with the aorta. There was no ingrowth of endothelial cells at the level of the pulmonary orifice (Fig 8, d).

**Discussion**

**Origin of Endothelial Cells**

The use of a species-specific antibody against endothelium\(^1\) proved to be essential for the description of early vessel formation in the wall of the developing heart. The anti-MB1 antibody also recognized precursor cells, allowing for the study of not-yet-lumenized vessels.\(^7,10,28\) Our results have shown that in the earliest stages (HH14 and HH15) the myocardial heart tube is
not yet vascularized. In subsequent stages, the heart tube is covered by epicardium in a way already described in detail in the literature.\textsuperscript{9-12} From our studies of both normal quail and chimeras, it could be deduced that the endothelial cells for the coronary vasculature closely followed the epicardial formation. The negative
results of our selective epicardial primordium transplants as to the development of endothelial cells strongly support the view that endothelial cells do not spontaneously differentiate within the subepicardial layer. Further data that support this view can be deduced from our results in normal quail embryos, in which the epicardial protrusions were at first completely devoid of endothelial cells or precursors (HH16), followed at HH17 by the first precursors in the epicardial primordium between the liver and sinus venosus.

An origin of coronary endothelial cells independent of the epicardial differentiation is supported by a chimera (embryo 0) that survived until stage HH19. This chimera showed a graft that was attached to the body wall. From this graft, the endothelial cells had colonized the epicardial primordium, as was to be expected for this stage. Furthermore, we have shown that the epicardial cells are positive for cytokeratins, whereas the endothelial cells and their precursors are negative in this respect, suggesting a different origin. The experiments of Mikawa and Fischman21 also favor an independent origin of endothelial cells. They showed by retroviral labeling of the epicardial organ that endothelial cells as well as smooth muscle cells and adipocytes migrate into the epicardium. The formation of clones suggested an origin of the clones from a single infected cell. The migration capacities of endothelial cells by inhibition studies using RGD peptides have been previously described.22 The subepicardial precursors and vessels were seen to contact the heart tube at the level of the sinus venosus, at the myocardium of atria and ventricle, and at the arterial pole. The characteristics and timing of these connections is discussed below.

**Sinus Venosus**

Sprouting of the sinus venosus to form the coronary veins has been described for various animal species.23-26 Whether the mechanism by which the first vascular contact between the liver sinusoids and the sinus venosus (HH17 to HH19) is made by a process that has been described as vasculogenesis (seeding by precursors as defined by Risau and colleagues29,30) or angiogenesis (sprouting from existing vessels as defined by Risau and colleagues29,30) is hard to determine even by our chimera studies. The impression exists that we always first detected precursors indicating the process of vasculogenesis. These precursor cells then form small vessels that seem to grow into the sinus venosus. This is supported by the observation that the sinus venosus is also lined by grafted quail endothelial cells. In contrast, data in previous literature that did not include the possibility of detecting endothelial precursors by immunohistochemistry or the use of chimeras favored the concept of the sprouting of vessels from the sinus.
venous to the subepicardial network. This mechanism is not supported by our data from normal quail and the chimeras.

**Myocardial Vascularization**

With regard to the vascularization of the myocardium, we have conclusive evidence that endocardial cells do not contribute to the intramyocardial vascular network as has been postulated in the literature. The chima experiments were essential for this conclusion because we were not able to distinguish between endocardially and subepicardially derived endothelial cells in the normal quail embryo. The only region that showed contact of endocardial and intramyocardial vessels was the right ventricular outflow tract. This implies that we have not found in our embryonic avian model evidence for extensive connections between the ventricular lumen and coronary vascular bed.

In the hatched embryo, where the graft was located in the atroventricular sulcus, there was a remarkably dense quail endothelial cell distribution of the myocardium, especially involving the inlet part of the right ventricle. It remains to be investigated whether this finding can bear relevance for a better understanding of the development of the right ventricle as well as the tricuspid valve. A similar interesting area that is always abundantly vascularized is the outflow tract septum of the heart. It is known that this septum develops relatively late in cardiogenesis and possibly has an extracardiac mesenchymal contribution. The concurrence of a possible extracardiac cellular contribution to the heart and endothelial ingrowth was also obvious at the atrioventricular sulcus area near the dorsal mesocardium, where endothelial cells were seen to move between the myocardium and the endocardial cushion tissue of the atroventricular canal. Whether this phenomenon bears relevance for atroventricular valve formation is not known.

**Arterial Orifice Level**

As shown before by our research group, the coronary arterial orifices are formed by a process of ingrowth into the aorta instead of the earlier postulated outgrowth. A renewed detailed study of the arterial orifice level in the normal quail embryo and the chimeras confirmed this earlier finding. We were, however, able to add some essential new data. First, ingrowth of endothelial cell strands is seen in all three aortic semilunar sinuses, amounting up to three or four strands in one sinus. This confirmed data from earlier literature postulating the presence of more sprouts. From these studies, however, only Waldo et al have described, on the basis of ink injections, that the process should be seen as ingrowth into rather than outgrowth from the aorta. Although our findings showed these strands to be present also in the so-called noncoronary or nonfacing sinus, it was confirmed that only two vessels showed a marked lumen. These were consistently connected to the facing semilunar sinuses of the aorta. Eventually, the nonluminated strands disappear.

We do not understand what factors govern the persistence of these two vessels in both aortic semilunar sinuses. Factors determining media formation, which have been described as preceding stabilization of the branching pattern, may play a role. The saddle-shaped orifice level is taken into account as well as the difference in the spatial relation of the periaortic cuffs and the aortic and pulmonary orifice. The periaortic vascular plexus that forms interconnected rings surrounding the aortic orifice and, in part, the pulmonary orifice can explain the potential possibilities for variations in the proximal branching pattern of the coronary arteries as seen in congenital heart malformations and occasionally as an isolated malformation.

**Relevance for Human Coronary Vascular Abnormalities**

With regard to our understanding of abnormal ventriculocoronary communications (fistulas), as seen in certain congenital heart malformations in the human, there was no evidence in our model of numerous embryonic connections. The only region that showed contact of the endocardium and intramyocardial vessels was the right ventricular outflow tract. The right ventricle is indeed more prone to congenital malformations (fistulas), but these are not exclusive for this region. If chick and human heart development is comparable, it can be deduced that fistulas are not remnants of normal embryonic connections but develop on the basis of, for example, abnormal hemodynamic parameters in the right ventricle during development.

Explanations for an abnormal connection of a coronary artery to a pulmonary artery are still lacking, although our studies have proven a difference in the relation of the periaortic cuff to the aortic and pulmonary orifice. A new finding from our present studies shows the periaortic cuff to also be present between the arterial orifices, providing a substrate for the described coronary abnormality with an intramural in-between course of a coronary artery. A possible mediating role for the neural crest–derived parasympathetic innervation that is seen in close contact both in space and time with the coronary vascular development needs to be considered.

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