Development of Proximal Coronary Arteries in Quail Embryonic Heart

Multiple Capillaries Penetrating the Aortic Sinus Fuse to Form Main Coronary Trunk

Katsumi Ando, Yuji Nakajima, Toshiyuki Yamagishi, Sadamu Yamamoto, Hiroaki Nakamura

Abstract—Studies have shown that the proximal coronary artery (PCA) develops via endothelial ingrowth from the peritruncal ring (PR) of the coronary vasculature. However, the details of PCA formation remain unclear. We examined the development of PCAs in quail embryonic hearts from 5 to 9 days of incubation (embryonic day [ED]) using double-immunostaining for QH1 (quail endothelial marker) and smooth muscle α-actin. At 6 to 7 ED, several QH1-positive endothelial strands from the PR penetrated the facing sinuses, and in some embryos, several endothelial strands penetrated the posterior (noncoronary) sinus. At 7 to 8 ED, the endothelial strands penetrating the facing sinuses seemed to fuse, forming a proximal coronary stem that was demarcated from the aortic wall by the nascent smooth muscle layer of the coronary artery. By 9 ED, two coronary stems were completely formed, and the endothelial strands previously penetrating the noncoronary sinus had disappeared. Observations demonstrate that during the formation of the PCA, endothelial strands from the PR penetrate the facing sinuses and then fuse, whereas those strands penetrating the noncoronary sinus disappear. Thereafter, the coronary artery tunica media demarcates the definitive PCA from the aortic media. (Circ Res. 2004;94:346-352.)

Key Words: coronary artery ■ development ■ quail embryo ■ QH1

During early heart development, a coronary circulation is absent, because the cardiomyocytes are in direct contact with the endocardium. After the formation of the d-loop heart (stage 14 to 15 in chick embryo, 2.5 embryonic day [ED]), the proepicardial organ develops from the dorsal mesocardium of the sinus venosus, migrates onto the surface of the cardiac tube, and gives rise to the primitive epicardium. After the formation of the epicardial covering of the heart, vascular buds or blood island–like structures, which arise in the epicardium, form in various species, including birds. In chick embryo, endothelially lined vascular anlagen appear in the epicardium at approximately stage 21 (3.5 ED). Coronary vascularization begins to occur at stage 32 (7.5 ED), first as venous sinusoids in connection with trabecular channels and second as coronary arterial vessels anastomosing with the venous sinusoids. Then a closed coronary system is completed by stage 41 (15 ED). Recent experiments with chick-quail chimeras and retroviral tracers have shown that not only coronary vessel endothelial cells but also smooth muscle cells originate from the epicardium. retroviral-tracer experiments have also made clear that the cells making up the coronary vessels originate from discontinuous colonies, suggesting that coronary vessels are established by vasculogenesis rather than by angiogenesis.

Several investigators have examined the origin of the proximal coronary artery stems and coronary orifices. A process often assumed to be involved in the formation of the proximal coronary trunk is one in which coronary arteries appear as endothelial sprouts from each coronary sinus, with the endothelial bud ultimately connecting with the peritruncal ring (PR) of the coronary vasculature in the subepicardial layer of the developing heart. Although this is a frequent assumption in the literature, sprouting of coronary arteries from the aorta has never been documented by micrographs showing an endothelial evagination. In fact, Dbaly et al failed to find a blind aortic evagination in rat embryos, whereas Bogers and colleagues found no coronary orifice without a connection to a proximal coronary artery. On the basis of such observations, it has therefore been concluded that the proximal segment of the coronary arteries develops by endothelial ingrowth from the PR rather than by endothelial outgrowth from the aorta. In the chick embryo, Aikawa and Kawano made the first report that at the onset of the formation of the proximal coronary arteries,

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multiple primitive coronary arteries originate from the right and left coronary sinuses and then decrease in number to form definitive coronary arteries on each side. Later, Poellmann et al. noted that multiple endothelial strands grow into the aorta from the PR at several sites and that the capillary network persists at only two of these sites to form definitive coronary arteries. At this stage, the first vascular smooth muscle cells come to surround the right and left coronary arteries (Hamburger-Hamilton stage 32; 7.5 ED). Notwithstanding the above observations, the details of the development of the coronary vascular system, including the formation of the proximal coronary arteries, remain unclear.

In the present study, we made detailed observations on the development of the main coronary trunks in quail embryos. For this, we used double immunostaining using an endothelial marker (QH1) and anti-smooth muscle α-actin (1A4) in serial sections cut parallel to the aortic orifice. In addition, using the confocal microscope, we observed the initial development of the endothelial strands (anlagen of the proximal coronary arteries) in the aortic wall.

### Materials and Methods

#### Quail Embryos

Fertilized eggs from the white egg strain of quail (Coturnix coturnix japonica; Nisseiken, Kobuchizawa, Japan) were incubated for 5 to 9 days at 37.8°C and 80% humidity. The number of embryos examined and their stages are indicated in Figure 1. Staged embryos were collected on ice-cooled PBS, and blood cells were washed out using cold PBS followed by 4% paraformaldehyde in PBS (both injected into the ventricle via a fine glass needle). Extirpated hearts were fixed additionally in 4% paraformaldehyde in PBS for 12 hours at 4°C. After extensive washing in PBS, aorticopulmonary regions were resected and embedded in paraffin. Then, 5-μm sections parallel to the aortic orifice were cut serially (Figure 2), transferred to glass slides, and subjected to immunostaining.

#### Antibodies

A monoclonal antibody, QH1 (mouse IgG1), which recognizes quail endothelial cells and hematopoietic cells, was obtained from the Developmental Studies Hybridoma Bank (Iowa City, Iowa). In addition to QH1, we used anti-smooth muscle α-actin (1A4) and anti-vimentin (H9251) antibodies in the immunostaining procedure.

### Table

<table>
<thead>
<tr>
<th>Embryonic Day</th>
<th>Number of penetrating endothelial strands (shown for each embryo)</th>
<th>(means)</th>
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</tr>
<tr>
<td>ED 9</td>
<td><img src="image5" alt="Diagram" /></td>
<td>0/1.0/1.0</td>
</tr>
</tbody>
</table>

* indicating an endothelial strand connecting between PR and aortic lumen

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**Figure 1.** Number of endothelial strands penetrating the aorta in each embryo examined at 5 to 9 ED. At 5 ED, there is no QH1-positive endothelial connection between the peritruncal ring and the aortic lumen. At 6 ED, QH1-positive endothelial strands begin to penetrate into right and left coronary sinuses. At 7 ED, a mean of >3 endothelial strands is penetrating into right and left sinuses, and in 3 out of 5 hearts, endothelial strands are penetrating the noncoronary sinus. By 9 ED, development of the right and left proximal coronary trunks has been completed, and no QH1-positive endothelial strands are penetrating the noncoronary sinus. At all stages examined, there is no QH1-positive endothelial strand penetrating the pulmonary trunk. A indicates aorta; L, left coronary sinus; N, noncoronary sinus; P, pulmonary artery; and R, right coronary sinus.

**Figure 2.** Schematic representations of quail embryonic heart (frontal view) to indicate the levels of the histological sections (a through c) shown in subsequent figures. Level a, across the aortic base; levels b and c, across outflow-tract cushion tissues (5 to 6 ED) or semilunar valves (7 to 9 ED). A indicates aorta; P, pulmonary trunk; RA, right atrium; LA, left atrium; RV, right ventricle; and LV, left ventricle.
Anti-smooth muscle α-actin (clone 1A4, IgG2a) was purchased from Sigma (St Louis, Mo). For double immunohistochemistry, we used TRITC-conjugated goat anti-mouse IgG2a and FITC-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, Ala) as secondary antibodies.

**Indirect Immunofluorescence Microscopy**

After deparaffinization, rehydration, and rinsing in PBS for 15 minutes, sections were blocked with 1% BSA in PBS for 30 minutes and then incubated with primary antibody mixture (1A4, ×400; QH1 [hybridoma supernatant], ×10 in blocking solution) in a moist chamber for 2 hours at room temperature. After rinsing in PBS, sections were incubated with secondary antibody mixture (×50 in blocking solution) for 1 hour. After additionally rinsing in PBS, sections were coverslipped using mounting medium (0.2 mol/L N-propylgallate in 10% PBS and 90% glycerol) and then examined under a conventional fluorescence microscope (BX60; Olympus) and photographed.

**Confocal Microscopy**

Truncal regions of 6 ED hearts were fixed in 4% paraformaldehyde in PBS. After rinsing in PBS, samples were treated with a graded series of sucrose in PBS (5% to 20% sucrose in PBS), embedded in OCT (Miles), and frozen on dry ice/acetone. Then, after 50-μm-thick sections had been cut on a cryostat, samples were mounted on glass slides, air dried for 30 minutes, and rinsed in PBS. Samples were blocked with 1% BSA/PBS for 30 minutes, stained with QH1 for 2 hours at room temperature followed by FITC-conjugated secondary antibody, and embedded in mounting medium. The resulting sections were examined using a BioRad Radiance 2000 laser scanning confocal imaging system fitted to a Nikon TE2000-U.

**Results**

**Development of the Proximal Coronary Arteries in Staged Embryos**

At approximately 5 to 6 ED, the aorticopulmonary septum was complete, but the septation of the proximal part of the outflow tract was still incomplete. At this time, subepicardial sinusoidal structures, which were encircled by QH1-positive cells (presumably endothelial cells), surrounded both the aortic and pulmonary trunks (Figures 3a1 and 3b1). Some mesenchymal cells subjacent to the QH1-positive endothelial cells of the subepicardial capillary network expressed both smooth muscle α-actin (SMA) and the QH1 epitope (not shown). Cells making up the aortic wall or the pulmonary trunk expressed SMA extensively (Figures 3a2 and 3b2). At this stage (ED 5), there was no apparent QH1-positive endothelial connection between the PR and the aortic lumen (Figure 1). Mesenchymal cells making up the aorticopulmonary septum also expressed SMA (asterisk in Figure 3b3).

At 6 ED, in three out of five embryos, QH1-positive endothelial strands connecting the PR to the aortic lumen
were found in the aortic wall facing the future left or right coronary sinus (arrowheads in Figures 4 and 1), much as described by Aikawa and Kawano.\textsuperscript{12} However, no QH1-positive endothelial strands were detectable in the wall of the pulmonary trunk in any of the ED-6 serial sections we examined. At this stage, although three out of five embryos showed QH1-positive endothelial strands penetrating the aortic wall of the right coronary sinus (the anlagen of the right coronary artery), two out of five embryos showed endothelial strands penetrating the aortic wall of the left coronary sinus (the anlagen of the left coronary artery) (Figure 1). At this stage, there were no apparent endothelial strands penetrating the aortic wall of the noncoronary sinus. Within the peripheral subepicardial endothelial network, SMA-positive cells (presumably smooth muscle cell progenitors) were observed subjacent to the endothelial cells (arrows in Figure 4).

At 7 ED, all 5 embryos we examined showed QH1-positive endothelial strands penetrating the aortic wall of both the right and left coronary regions (the facing sinuses). In addition, in three out of five embryos, QH1-positive endothelial strands were seen penetrating the aortic wall facing the noncoronary cusp (*N in Figure 5); indeed, endothelial strands with an apparent lumen were observed penetrating the aortic wall of the noncoronary sinus (Figure 5d). At this stage, the endothelial strands in the aortic wall of both the right and left coronary sinuses were greater in number (Figure 1) and had an apparent vascular lumen, as described by Aikawa and Kawano.\textsuperscript{12} These endothelial strands penetrating the aortic wall facing the right and left coronary sinuses seemed to become fused to each other, with the result that a reticular structure consisting of an endothelial plexus with decidual tissues could be clearly seen in the aortic wall (open arrowhead in Figure 5e). Such a fusion of the endothelial strands penetrating the aortic wall facing the sinuses may contribute to the generation of a single proximal coronary trunk. Certainly, we did not find such a QH1-positive endothelial reticular structure with decidual tissues in the noncoronary sinus region, where a definitive (permanent) main coronary trunk does not develop. In addition, thick, markedly SMA-positive structures could be seen to surround the developing left and right proximal coronary trunks that are penetrating the aortic wall (arrows in Figure 5). On the other hand, only weak SMA staining was observed in cells surrounding the endothelial strands penetrating the noncoronary sinus (Figure 5).
At 8 ED, all embryos possessed a single right coronary trunk, and four out of five possessed a single left coronary trunk (the remaining embryo had two left proximal coronary arteries). At this stage, a thin QH1-positive endothelial strand penetrating the noncoronary sinus was seen in three out of five embryos (Figure 1). By 9 ED, the right and left proximal coronary trunks had been completed in all 5 embryos examined (Figures 1 and 6). At this stage, there was no QH1-positive endothelial strand connecting to the noncoronary sinus, and the right and left coronary orifices were manifest as a single, large concavity surrounded by a thick SMA-positive smooth muscle cell layer (tunica media of the coronary artery) (arrowheads in Figure 6). SMA-positive smooth muscle layers also surrounded the peripheral coronary vessels (arrows in Figure 6).

Confocal Microscopic Observation of the Initial Formation of Endothelial Strands

It has been reported before that coronary arteries do not grow out of the aorta but grow into the aorta from the PR. However, details of the initial formation of endothelial strands remain unclear, possibly because of methodological limitations, because previous observations were made using conventional histological sections. Therefore, in our examination, we cut thick (50-μm) sections of the trunical region in 6 ED embryos (N=5), stained them with QH1 antibody, scanned them using a laser confocal microscope every 2 μm, and reconstructed the images. Confocal microscopic observations revealed that QH1-positive discontinuous mesenchymal cells (presumably endothelial progenitors), which did not make contact either with the PR or with the aortic endothelium, were present within the aortic wall at sites where the endothelial strands later develop (arrows in Figure 7). In addition, some QH1-positive cells were seen connecting either with PR or with both PR and the aortic lumen (arrowheads in Figure 7), although we found no QH1-positive mesenchymal cells connecting with the aortic lumen alone. Furthermore, as described by Bogers et al. we found no case in which the coronary orifice did not connect to the PR. These observations suggest that the initial formation of endothelial strands is established from QH1-positive mesenchymal cells in the aortic wall and that the formation of endothelial strands progresses in an outside-to-inside (PR-to-aortic) direction.

Discussion

Morphological Mechanisms Involved in the Formation of Proximal Coronary Segments

In the present study, we have shown that multiple endothelial strands are penetrating the three aortic sinuses, including the noncoronary (posterior aortic) sinus, at the onset of the formation of the proximal coronary segments. Waldo et al., using an India ink injection method, reported that multiple coronary vessels connect to the right, left, and rarely the noncoronary sinus in the early development of the proximal coronary arteries in the chick embryo. They also noted that some of these multiple coronary arterial vessels had apparent vascular lumina. Several investigators have described that whereas multiple endothelial strands of the coronary arterial anlagen from the PR connect to the aorta, only one channel survives in each of the two sinuses to become the definitive right and left proximal coronary arteries and their orifices. In our observations, endothelial strands penetrating the aortic wall facing either the right or left coronary sinus seemed to become fused to each other. As a consequence, a reticular structure consisting of endothelial strands (plexus) with decidual tissues was clearly visible in the aortic wall where the future main coronary trunk and its orifice would later develop. On the other hand, we did not find such a reticular structure in the noncoronary sinus. As to why the endothelial strands penetrating the noncoronary sinus disappear rather than persisting to form a definitive proximal coronary artery, one possibility is that the endothelial strands are too few and their penetration too sparse for such fusion to occur (Figure 1). Although apoptotic cells have been observed in close spatial and temporal association with the developing coronary arteries and their orifices, we could not find typical apoptotic cells within the decidual tissue (not shown). Therefore, in addition to apoptosis, unknown mechanisms involved in the formation of proximal coronary trunk from the multiple endothelial strands at a proper site might be considered. Other experiments have suggested that the formation of the tunica media is the determining factor if multiple coronary vessels penetrating the aortic wall are to survive to become the
definitive proximal coronary trunk. In the present study, a thick SMA-positive tunica media belonging to the developing coronary artery was observed surrounding the right and left proximal coronary arteries penetrating the aortic wall, whereas only weak anti-SMA immunoreactivity was seen in cells surrounding the endothelial strands penetrating the noncoronary sinus (Figure 5). It seems likely that the establishment of the coronary artery tunica media is important to demarcate the definitive proximal coronary segments from the aortic media. Other investigators have suggested that whereas parasympathetic ganglia derived from cardiac neural crest cells do not directly contribute to coronary vessel elements, they significantly affect the organization and development of coronary arteries. Our observations, taken together with those of others, seem to offer the following new insights into the morphological mechanisms involved in the generation of proximal coronary arteries and their orifices: (1) multiple endothelial strands penetrate the aortic wall at several sites; (2) the right and left coronary stems develop (probably established by fusion of the endothelial strands) and the endothelial strands connecting the noncoronary sinus disappear; and (3) the coronary artery tunica media demarcates the definitive proximal coronary arteries from the aortic media.

**Initial Formation of the Endothelial Strands Penetrating the Aortic Wall**

Several investigators have noted that coronary arteries grow into the aorta from the PR, with Waldo et al concluding that the process is an angiogenic event. Retroviral-tracer experiments have revealed that the coronary vessels are established by an embryonic process of vasculogenesis. Two basic mechanisms have been proposed in the literature to explain how endothelial tubes form during embryogenesis (vasculogenesis and angiogenesis). Angiogenesis is the sprouting of new vessels from existing vessels, whereas vasculogenesis involves the de novo differentiation of angioblasts and organization into a vascular plexus, followed by remodeling into definitive vessels. A third process may involve congruent angiogenesis and vasculogenesis.

Quail-chick chimeras have previously been constructed to trace the fate of the proepicardial organ, and their use has shown that the endothelial cells of the proximal coronary arteries originate from the proepicardium-derived subepicardial mesenchyme. Our confocal microscopic observations showed that QH1-positive discontinuous endothelial progenitors (possessing a mesenchymal phenotype), which did not make contact either with the PR of the coronary vasculature or with the aortic endothelium, were present within the aortic wall at sites where the endothelial strands would later develop (Figure 7). There is no direct evidence to indicate whether such QH1-positive mesenchymal cells in the aortic wall originate from proepicardium-derived subepicardial mesenchyme and will later take part in the development of the endothelial strands. Our confocal microscopic observations, together with other observations, suggest that the endothelial strands penetrating the aortic wall are derived from the QH1-positive mesenchymal cells (probably proepicardium-derived endothelial progenitors), and the formation of the endothelial strands seems to be established by vasculogenesis or vasculogenesis plus angiogenesis.

**Relation Between Multiple Endothelial Strands and Human Coronary Artery Malformations**

There are several types of primary congenital abnormalities involving the proximal coronary arteries (eg, high takeoff, multiple ostia, absent proximal ostium, single ostium, hypoplastic proximal coronary artery, and coronary artery from the posterior aortic sinus). The number of coronary ostia varies, and a common feature in the ostium of the right coronary artery is that in approximately half of all hearts, a second small orifice is present in the right coronary sinus, from which the infundibular artery arises. In addition, an intramural coronary artery with a variable course can be present in patients with transposition of the great arteries. One possibility is that an inappropriate or incomplete fusion of the multiple endothelial strands may contribute to the generation of these abnormal proximal coronary arteries.

The usual interpretation of conventional histological observations of the development of the proximal coronary trunk is that whereas multiple coronary arterial buds grow from both aorta and pulmonary sinuses, only two buds, right and left, hollow out, increase in length, and connect to the right and left vascular networks, respectively, allowing coronary arteries to be formed. This simplistic developmental scheme might seem to facilitate our understanding of all known coronary artery abnormalities, including an aberrant origin of the left coronary artery from the pulmonary trunk (a condition known as Bland-White-Garland syndrome). However, we (in this report) and others have failed to find any endothelial buds originating from the pulmonary sinuses. We believe the aberrant origin of the coronary artery from the pulmonary trunk may be attributable to an abnormal development of the endothelial strands penetrating the pulmonary trunk from the PR rather than to the persistence of a vessel arising from the pulmonary trunk. We also suggest that unknown mechanisms that inhibit the formation of the endothelial strands penetrating the pulmonary trunk from the PR might be considered in the initial formation of the normal coronary trunk.

In the present report, we have described the morphological mechanisms involved in the initial formation of the proximal coronary arteries, as follows: (1) multiple endothelial strands penetrate the aortic wall at several sites; (2) fusion of the multiple endothelial strands occurs at the facing sinuses, while at the same time the endothelial strands penetrating the noncoronary sinus disappear; and (3) a coronary artery tunica media develops, and this demarcates the definitive proximal coronary arteries from the aortic media.

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References

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