Epicardial Outgrowth Inhibition Leads to Compensatory Mesothelial Outflow Tract Collar and Abnormal Cardiac Septation and Coronary Formation

Adriana C. Gittenberger-de Groot, Mark-Paul F.M. Vrancken Peeters, Maarten Bergwerff, Monica M.T. Mentink, Robert E. Poelmann

In the present study, we investigated the modulatory role of the epicardium in myocardial and coronary development. Epicardial cell tracing experiments have shown that epicardium-derived cells are the source of interstitial myocardial fibroblasts, cushion mesenchyme, and smooth muscle cells. Epicardial outgrowth inhibition studies show abnormalities of the compact myocardial layer, myocardialization of cushion tissue, looping, septation, and coronary vascular formation. Lack of epicardial spreading is partly compensated by mesothelial outgrowth over the conotruncal region. Heterospecific epicardial transplant is able to partially rescue the myocardial development, as well as septation and coronary formation.

The important role of the proepicardial organ (PEO) and its epicardium-derived cells (EPDCs) in cardiac development has recently been recognized. Data from both chicken-quail chimeric techniques,1–3 retroviral lineage tracing,4 and in vitro data5 show that EPDCs are the source of coronary smooth muscle cells, cardiac interstitial fibroblasts, and mesenchymal cells in the atrioventricular cushions. Chimeric studies6,7 showed that the coronary endothelial cells (ECs) originate from the endothelium of the liver region.

Our present study applying microsurgery to inhibit outgrowth of the PEO was set up to investigate the possibility that coronary ECs were not only derived from the sinus venosus region but could also be recruited under abnormal circumstances from the pharyngeal arch region, which explains aberrant coronary artery origin in neonates.8 The second aim was to investigate the role of EPDCs in the myocardium, the endocardial cushions, and the coronary vasculature.

Material and Methods

We used embryos of the Japanese quail (Coturnix coturnix japonica) and the White Leghorn chicken (Gallus domesticus) for two sets of experiments. In the first set, inhibition of outgrowth of the epicardium in quail embryos was obtained as described by Männer.2 In 13 embryos, complete or partial absence of outgrowth of the epicardium from the venous pole was found (Table), which was confirmed in 8 embryos by cytokeratin staining. The second set consisted of 10 chicken host embryos (HH15) in which, after inhibition of outgrowth of the host PEO, a quail PEO (HH15 to HH17) was implanted, resulting in a 1-day delay in outgrowth. In all embryos (4n:HH29, 2n:HH30, and 4n:HH35), quail epicardium covered the greater part of the heart. Embryos were serially sectioned and subjected to standard immunohistochemical procedures6,7 including the TUNEL technique for detection of apoptosis.10

Results and Discussion

Epicardial Outgrowth Inhibition

No living embryos beyond HH32 were harvested after epicardial outgrowth inhibition. In most cases (Table), there was a remarkable compensatory outgrowth of the mesothelium from the pharyngeal arch area over the conotruncal region. Normally, the epicardium reaches up to the arterial-myocardial borderline.9 In outgrowth-inhibited embryos, there is a collar of cytokeratin-positive mesothelium that not only covered the great arteries but also the myocardial outflow tract up to the level of the inner curvature (Figure, a and b).

In the experimental embryos, the compact layer of the ventricular myocardium, lacking epicardium, proved to be abnormally thin (compare Figure, c and e with d and f). Focally, there was myocardial necrosis. The thin myocardium did not show altered apoptosis compared with normal.11 The cardiomyocytes were rounded and not well organized in contrast to normal myocardium.12 The outflow tract myocardium, covered by the collar, did not show marked abnormalities. These findings point to a role for the early intramyocardial EPDC population1 in inducing normal myocardial development. This is supported by data from α4 integrin13 and vascular cell adhesion molecule-114 knockout mice with a similar myocardial phenotype. In addition, because no coronary network is formed, myocardial undernutrition might play a role.12

There were variable degrees of abnormal ventricular inlet and outflow tract septation. The heart tube was still primitive, with deficient looping and a wide inner curvature (Figure, c), and presented in all cases with a double-inlet, double-outlet configuration (Figure, c and i). In 3 of 13 cases, there was fusion of the endocardial outflow tract ridges. All others still presented with a common arterial trunk. Myocardialization of these ridges, normally taking place from stage 31 onward,10 was absent (Figure, j and k). The deficient atrioventricular cushions (Figure, c and e) had not fused to form a tricuspid and a mitral orifice but presented as a common atrioventricular canal. Formation of the interventricular septum was deficient (Figure, i) or absent (Figure, c), which concurs with the aforementioned mice knockout data.14 Because none of the embryos survived beyond HH32, we could not evaluate the definitive heart malformations. The malformations correlate with early stages of the recently described GATA cofactor FOGER2+ hearts.15 In the FOGER2+ phenotype, there is epicardial formation with lack of coronary formation, which
could point toward a defective endothelial outgrowth from the transverse septum in a deficiently differentiated epicardium.

Our epicardial ablation embryos showed obvious deviations from normal coronary endothelial plexus formation. In most cases, the small vessels arising from the sinus venosus used the small patch of epicardium at the venous pole to reach the ventricular myocardium, where in contrast to normal development, connections with the ventricular lumen were established. The remainder of the myocardium lacked coronary vasculature.

At the arterial pole, a compensatory mechanism was seen in which in the submesothelium or adventitia of the pharyngeal arch arteries, an extensive endothelial vessel plexus, reached the arterial-myocardial border (Figure, b and g). In two cases, this network penetrated the aortic wall forming a coronary arterial orifice (Figure, b, and Table). These data support the potential for origin of coronary arteries from thoracic arteries in humans.8

**Epicardial Rescue Experiments**

Before HH29, an outflow tract mesothelial collar was seen comparable to the inhibition series. At HH35, the quail-derived donor epicardium had moved up to the myocardial-arterial borderline. We assume that this change is due to the shortening and remodeling of the outflow tract as a relatively late process in cardiac development, as was also suggested by Münzer.2 Quail EPDCs participated in all cell types as described in normal development.1

Our findings are unique in that we show that epicardial grafting rescued the cardiac phenotype. The compact myocardium was well developed already, before functional coronary vascularization was established (Figure, f). The cushions of both outflow tract and atrioventricular levels had fused, and myocardialization had taken place and cardiac septation was normal.

However, in the HH35 embryos, we found two cases with coronary abnormalities. In one, a coronary artery had connected to the pulmonary orifice, as in the Bland White Garland syndrome.8 In the other, the connection of both main coronary arteries to the aorta was missing but was replaced by two ventriculo-coronary communications (fistulae). This supports an important clinical notion that fistulae, accompanying pulmonary atresia without ventricular septal defect,16 may develop as a primary structural coronary abnormality before obliteration of the pulmonary orifice, instead of being caused

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### Table: Quail Embryos Studied After Epicardial Outgrowth Inhibition

<table>
<thead>
<tr>
<th>Inhibition</th>
<th>Harvest</th>
<th>Outgrowth</th>
<th>Outflow Collar</th>
<th>Outflow Tract</th>
<th>Sinus Venous</th>
<th>Myocardium</th>
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<tr>
<td>HH14</td>
<td>15</td>
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Experimental age varied from HH14 to HH17, survival from HH25 to HH32. Comparison of harvest and outgrowth stages of PEO shows degree deficiency of epicardial covering. Partial myocardialization of the *atrioventricular and †outflow tract cushions; ‡lumen connection to aorta and §right atrium.
secondarily by increased right ventricular pressure resulting from pulmonary obstruction.

**Acknowledgments**

This work was supported by grants D96.017 and 99.022 from the Netherlands Heart Foundation.

**References**


**Key Words**: epicardium  ■  coronary vasculature  ■  myocardial differentiation  ■  ablation  ■  chimerization
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Circ Res. 2000;87:969-971
doi: 10.1161/01.RES.87.11.969

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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