Coronary Artery and Orifice Development Is Associated With Proper Timing of Epicardial Outgrowth and Correlated Fas Ligand Associated Apoptosis Patterns

Ismail Eralp, Heleen Lie-Venema, Marco C. DeRuiter, Nynke M.S van den Akker, Ad J.J.C. Bogers, Monica M.T. Mentink, Robert E. Poelmann, Adriana C. Gittenberger-de Groot

Abstract—The proepicardial organ provides differentiated cell types to the myocardial wall and facilitates coronary development. Ingrowth of the coronary arteries into the aorta has recently been linked to apoptosis. This study was set up to examine the effect of an inhibition of epicardial outgrowth on apoptotic patterning and coronary development. Epicardial outgrowth was blocked at HH15–17 in quail embryos, which survived until HH25–35 (n=33). Embryos with complete inhibition of outgrowth did not survive after HH29. These embryos presented with thin compact myocardium, devoid of vessels. In embryos with delayed epicardial outgrowth the phenotype was less severe, and surviving embryos were studied up to HH35. In these embryos, myocardial vascularization was poor and apoptosis in the peritruncal region at HH30 was diminished. Embryos at HH35 displayed an abnormal coronary network and absent coronary orifices. In a further set of experiments (n=10), outgrowth was inhibited in chicken embryos at HH15, followed by transplantation of a quail proepicardial organ into the pericardial cavity to rescue cardiac phenotype. These chimeras were studied at HH29 and HH35. Myocardial development was restored; however, in 3 of 4 embryos (HH35), the coronary orifices were absent. Examination of double stainings of quail-chicken chimeras revealed that EPDCs produce Fas ligand as an apoptotic inductor at sites of coronary ingrowth. In the absence of proper timing of epicardial outgrowth, myocardial development and vascularization are disturbed. Also apoptosis in the peritruncal region is diminished. During later development, this leads to defective or absent connections of the coronary system to the systemic circulation. (Circ Res. 2005;96:000-000.)

Key Words: coronary arteries ▪ myocardium ▪ epicardium ▪ apoptosis ▪ development

In early embryonic life, the primitive heart tube consists of two layers, the myocardium and the endocardium. During development, a third layer, called the epicardium, is formed. Epicardial cells are derived from the proepicardial organ (PEO), which protrudes as a cauliflower-like structure from the mesothelial lining of the body cavity near the sinus venous and primitive liver, toward the inner curvature of the heart. The villi of the PEO reach the heart at the atrial side and the epicardial cells start to spread over the naked heart tube, until they eventually cover the myocardium completely.1–3 Between the epicardium and the myocardium, a subepicardial layer develops. Epicardium-derived cells (EPDCs), which detach from the epicardium by epithelial to mesenchymal transformation (EMT) make up this subepicardial mesenchyme. Both perihepatic endothelial cells from the dorsal mesocardium as well as EPDCs migrate into the subepicardial layer and produce extracellular matrix components. The subepicardial mesenchyme is most prominent at the atrioventricular (AV) and interventricular grooves. In contrast, the epicardial mesothelium remains in close contact with the free myocardium of the atria and ventricles. Coronary vessel network develops within the subepicardial mesenchyme. Initial endothelial network is in open contact with the sinus venous and starts growing over the heart encircling the AV groove toward the ventral side. The coronary endothelial plexus reaches the ventriculoarterial junction and forms a peritruncal ring. From here, vessels grow into the aorta and into the right atrium. This ultimately results in two coronary arteries that connect to the aorta. Part of the EPDCs migrate into the underlying layers to differentiate into several cell types. Smooth muscle cells of coronary arteries, myocardial and subendocardial interstitial fibroblasts, and a subpopulation of the mesenchymal cells of the endocardial cushions are derived from EPDCs. Several mouse models (VCAM-1, α4 integrin, and FOG2...
null mutants as well as our avian models have shown that coronary vessel formation is severely disturbed when the epicardium fails to develop properly. EPDCs that have migrated into the myocardial layer and differentiated into interstitial fibroblasts have been hypothesized to provide for a signaling function in myocardial development. Similarly, epicardially regulated signaling has been hypothesized to be related to apoptosis, which accompanies outflow tract remodeling and coronary ingrowth into the myocardium of the outflow tract region. Signaling through Fas and Fas ligand (FasL) has already been reported in the developing avian heart as a pathway of apoptosis. Another study reports that FasL can induce apoptosis in human coronary endothelial cells in vitro. The role of apoptosis could become clearer by studying the effects of absent or disturbed epicardial contribution. In the present study, inhibition and adjusted chimera techniques were used to determine the role of EPDCs in coronary vascular development and also to establish the role of FasL in this process.

Materials and Methods

White Leghorn chicken embryos (Gallus domesticus) and Japanese quail embryos (Coturnix coturnix japonica) were used and staged according to the criteria of Hamburger and Hamilton (HH). Normal controls consisted of developmental series of quail and chicken embryos from stages HH29 to HH35.

Inhibition of Epicardial Outgrowth

Outgrowth of the PEO in quail embryos (HH15 to HH18) was inhibited by a piece of egg shell membrane between the PEO and the heart tube, as described by Männer. After reincubation at 37.5°C (80% humidity), embryos were isolated at stages HH25 to HH35. We distinguished in the 33 harvested embryos between either a complete inhibition (n = 15) or a partial inhibition (n = 18). The embryos with partial inhibition were mechanistically considered to have a delay in epicardial outgrowth.

Rescue After PEO Inhibition

Rescued embryos were produced as quail-chicken chimeras, by transplanting a quail PEO of HH15–17 into the pericardial cavity of a chicken host of equivalent age, after inhibiting host PEO outgrowth. After reincubation, the embryos were isolated at stages HH29 to HH35.

Preparation of the Chicken-Quail Chimeras

After incubation at 37.5°C (80% humidity) for ~3 days, quail embryos ranging from HH15 to HH18 were used as donors. The chicken host embryos were incubated for ~3 days, and ranged from HH15 to HH18. A quail transplant was inserted into the pericardial cavity of the chicken, which could be reached through the naturally existing body wall hiatus at this stage. The transplant was placed beneath the heart tube adjacent to the sinus venous or in the inner curvature of the heart loop. After reincubation, the embryos were isolated at stages HH29 and HH30.

Immunohistochemistry

Serial sections were subjected to standard immunohistochemical procedures, including the TUNEL (Roche) staining for apoptosis. For the detection of quail cells in our chimeras, we used an anti-quail nuclear antibody (QCPN, Hybridoma Bank) diluted 1:2, and a quail endothelial marker (QH1, Hybridoma Bank), diluted 1:500. To analyze myocardial and vascular development, we used several specific markers, eg, the anti-actin muscle marker HHF35 (Dako) (1:500), the anti–quail-endothelial antibody QH1 (1:500), and the smooth muscle cell marker 1A4 (Sigma) (1:3000). Whole-mount cytokeratin (Dako) staining was performed to analyze epicardial covering of the heart. For the double stainings, sections were incubated overnight with the first primary antibody (aFasl: 1:200, Santa Cruz Biotechnology). Subsequently, they were incubated with a biotin-labeled secondary antibody for 1 hour, followed by TRITC-labeled avidin for 1 hour. Sections were incubated for 2 hours with the second primary antibody (QCPN 1:12) and incubated with secondary FITC-labeled antibodies. Sections were mounted with Prolong (Molecular Probes) and analyzed by confocal microscopy.

Analysis of Apoptosis Frequency and Morphometry

We measured the volumes of total myocardium, the outflow tract cushions, and the atrioventricular cushions. We also measured the apoptotic volumes of these specific regions while defining the perirterial ring as a separate region. We used the volume counting method according to Cavalieri. In short, the number of points on a grid hitting the tissue of interest on a series of sections was counted. Volumes could be calculated from the counted numbers by Cavalieri’s formula:

\[ V = \frac{1}{a^d} \sum P \cdot M \]

In this formula, V is the volume in mm³, \( \Sigma P \) is the total number of counted points, M is the magnification, a is the point area in mm², and d is the distance between the counted sections in mm.

Quail hearts (HH30) with a delay in epicardial outgrowth were compared with normal hearts of the same stage. The apoptotic volumes obtained in the delay group were normalized for differences in volumes. Results were compared statistically using Student t test.

Results

To analyze the regulatory role of EPDCs in myocardial and coronary development, three sets of experimental embryos were generated. We used quail embryos in which we blocked PEO outgrowth and chicken embryos in which inhibition of epicardial outgrowth was rescued by transplantation of an exogenous quail PEO. These two models show a range of heart malformations that illustrate the importance of properly timed epicardial behavior for the development of the coronary system and the myocardial architecture. Lastly, quail-chicken chimeras were generated to determine whether the proapoptotic factor FasL was produced by EPDCs.

Inhibition of Epicardial Outgrowth

We obtained 33 embryos in which epicardial outgrowth was completely (n = 15) or partially (n = 18) inhibited. The embryos with partial inhibition were mechanistically considered to have a delay in epicardial outgrowth.

In embryos with complete inhibition, the malformations were severe and as a result embryos did not survive after HH30. Whole-mount staining with the mesothelial marker anti-cytokeratin showed naked hearts (Figure 1a). In these hearts, there was compensatory outgrowth of cytokeratin-positive cells, earlier referred to as a mesothelial collar, over the myocardium of the outflow tract (Figure 1a through 1c).

Cardiac looping and septation were disturbed to a variable extent. All cases presented with a double-inlet to double-outlet configuration. In most embryos, a common arterial...
trunk was found and AV cushion formation was deficient or absent in all embryos (Figure 1b). Interventricular septation was disturbed. The compact myocardium and trabeculated myocardium in these embryos were abnormally thin (Figure 1d through 1g).

Coronary development was severely disturbed. After formation of an initial coronary endothelial plexus sprouting from the sinus venosus, no vessels had penetrated the myocardium (Figure 1h through 1k).

Delay in Epicardial Outgrowth; Effects at HH30
In partially inhibited embryos (n=18), the epicardial cells had reached the heart and grown over the myocardium, but because the eggshell membrane blocked the PEO initially, epicardial outgrowth was delayed by approximately 1 day.

We analyzed embryos at different stages, HH30 (n=10) and HH35 (n=8), to study both myocardial vascularization and the development of the coronary orifices, respectively.

In the embryos of stage HH30, myocardial architecture was normal (Figures 1d and 2c). However, volume measurements showed that the myocardial volume was significantly reduced by 47% (P<0.05) in embryos with delayed epicardial outgrowth compared with normal embryos (Table). Moreover, myocardial vascularization was severely disturbed. Immunohistochemical staining with the QH1 antibody showed that only the myocardium that was covered by epicardium with a properly developed subepicardial mesenchyme was vascularized correctly. In other parts, where the subepicardial mesenchyme was missing, vascularization was very poor (Figure 2).

Apoptosis Frequency and Morphometry
To study the relation of epicardial development with apoptosis in the peritruncal ring, we performed a TUNEL staining on embryos of stage HH30, just before coronary ingrowth. Apoptosis was markedly diminished in embryos with disturbed epicardial outgrowth (Figure 3 and Table 1). Apoptotic volumes of various areas of the heart were measured showing that apoptosis in the peritruncal ring was reduced by 83% (P<0.001), in the outflow tract myocardium by 56% (P<0.01) and in the outflow tract cushions by 88% (P<0.05). After normalization for myocardial volume, apoptosis in the peritruncal ring was reduced by 71% (P<0.01) and in the outflow tract cushions by 84% (P<0.05) (Table).

We did not only examine the incidence of apoptosis, but also the location of the apoptotic cells, concentrating on the ingrowth sites of the coronary arteries. In the control em-
bryos, we observed clusters of apoptotic cells at sites where the main stems of the coronary arteries invade the aorta. Around the pulmonary artery, we found only small numbers of randomly scattered apoptotic cells. In contrast, in the embryos with a delay in epicardial outgrowth, we observed not only reduced apoptosis, but also that the apoptotic cells were randomly located. Nevertheless, there was more apoptosis around the aorta than the pulmonary trunk.

**Rescue of Epicardial Inhibition**

To study whether the phenotype, observed in embryos with disturbed epicardial outgrowth, could be rescued, we created an experimental set in which transplantation of an exogenous PEO was performed after inhibition of the endogenous PEO. Thus a delay in epicardial outgrowth without the loss of epicardial cells could be induced. In this set, the obtained embryos (n=10) showed complete epicardial covering that was of mixed chicken and quail origin.

Myocardial development was restored to normal already before coronary vascularization was established (Figure 4a). The endocardial cushions were well developed and myocardialized. Scattered quail-derived EPDCs were seen throughout the myocardium of the ventricles and atria. No EPDCs were found in the AV cushions of the younger embryos (HH29/HH30; n=6), whereas the older embryos (HH35; n=4) showed abundant presence of EPDCs in the mitral and tricuspid valve leaflets (Figure 4b through 4d). Only two cases presented with a cardiac abnormality.

However, study of the proximal coronary stem development in the four older embryos (HH35), showed, that 3 of 4 embryos had abnormalities in their coronary-to-aortic connections. One case had no coronary connections to the aorta. Compensatory ventriculocoronary-arterial connections (VCACs or fistulae) had developed, one growing into the heart from the transplanted. The subepicardial VCACs were surrounded by cells, which expressed smooth muscle actin (Figure 4j). In the second embryo, we found only one atretic coronary artery connecting with a pinpoint orifice into the aorta at the left sided sinus (Figure 4e). In the third embryo, the coronary branches of the left coronary artery, the left

**Figure 2.** Sagittal sections of quail hearts (HH30) stained for quail endothelium (QH1 brown). a, Section of the right ventricle (RV) of a normal heart with evident presence of coronary vessels in the compact myocardium (CM) and in the ventricular septum (VS). Arrows indicate capillaries. b, Section of the RV of a quail heart with inhibition (inh) of epicardial outgrowth, with remarkably less endothelial cells compared with normal hearts. c, Section through apex of a heart (HH30) with inhibition, with myocardium is partly covered with normal epicardium (EP). Arrow indicates border of normal epicardium and epicardium without development of a subepicardial layer. CM, with a normal subepicardial layer, is penetrated by coronary vessels (arrows in d), whereas the abnormal myocardium with hardly any formation of a subepicardial layer is nearly devoid of vessels (e). LV indicates left ventricle; PT, pulmonary trunk. Scale bars=250 μm in a through c; 50 μm in d and e.
Production of Fas Ligand by EPDCs

We obtained six chicken embryo hearts (HH30), which were extensively populated by graft-derived quail cells. Double-stainings with the quail nuclear marker QCPN and the antibody against the intracellular component of FasL allowed us not only to locate the quail EPDCs, but also to determine whether these cells produced FasL. In all hearts, quail EPDCs were found that produced FasL. These cells were specifically found at sites of coronary ingrowth (Figure 5a through 5d). The stainings show that not only the EPDCs produced FasL, but also the graft-derived endothelial cells. The EPDCs and graft-derived endothelial cells at other sites were all negative for FasL (Figure 5e).

Discussion

This study confirms our earlier findings that after complete inhibition of epicardial outgrowth, embryos die due to structural and severe myocardial and vascular abnormalities. The myocardium does not develop and no endothelial capillary network is formed. Cardiac looping and cushion formation are also insufficient to form a functional heart.17 Tevosian et al15 presented a model with a comparable phenotype in which the FOG-2 gene, a cofactor of the GATA-family (GATA1–6) of transcription factors, is essential for EMT. These mice displayed disturbed vascularization and myocardial maturation. Reexpression of FOG-2 restores this phenotype.15

Similar to this genetic-model, transplanting a quail PEO into the pericardial cavity of a chicken embryo after inhibiting host epicardial outgrowth results in a rescue of myocardial development17 (this study). However, the transplanted PEO does not attach immediately to the myocardial heart tube and consequently a delay in epicardial outgrowth occurs. The delay is visible in the covering of the outflow tract, which contains a mixed population of chicken-derived arterial epicardium and quail PEO-derived cells, whereas in normal embryos or chimeras without outgrowth inhibition, the outflow tract epicardium at HH30 is made up solely by PEO-derived cells.3 In our rescued embryos, this state of development is reached only just before stage HH35 (not shown). A similar observation was made by Männner in a quail-chicken chimera model.12 The mixed population contains PEO-derived donor cells and arterially-derived epicardial cells. This is a result of compensatory outgrowth of arterially-derived epicardium, also seen in embryos with a complete inhibition of PEO outgrowth, where a collar of arterial epicardium grows over the proximal outflow tract.17 This arterial epicardium, with specific immunohistochemical characteristics, has also been described by Perez-Pomares and colleagues.29 A novel finding in our rescued embryos is the occurrence of coronary vascular abnormalities such as absent connections to the aorta and fistulae in the myocardial wall, indicating that proper timing of epicardial outgrowth is
essential for correct connection of the coronary stems to the aorta.

Earlier work demonstrated that proper development of the main coronary stems is established through ingrowth of endothelial cells into the myocardial wall and the aorta. A relation between apoptosis and coronary ingrowth was recently reported. Another study, using the PEO inhibition model demonstrated that, when epicardial outgrowth is delayed, apoptosis in the outflow tract is diminished, specifically during the stages at which the coronary vessel network connects with the systemic circulation. Watanabe and colleagues implied an important function of apoptosis in shortening and remodeling of the outflow tract. Based on the findings, reported both by Rothenberg et al and Velkey and Bernanke, we propose an experimental model in which delayed epicardial outgrowth causes coronary misconnection through diminished apoptosis. Secondary consequences are compensatory development of fistulae and abnormal remodeling of the vessel network.

To test our hypothesis, we interfered in epicardial outgrowth and harvested embryos at stages HH30 and HH35. Stage HH30 was to study apoptotic patterns in the outflow tract and subaortic region, because at that stage, a peak in apoptosis occurs in this region. Specifically in the peritruncal region, apoptosis was significantly diminished in embryos with PEO inhibition, supporting the hypothesis that proper timing of epicardial outgrowth is essential for normal levels of apoptosis.

Not only apoptosis was abnormal in these embryos. Parts of the myocardium that lacked epicardium with a proper subepicardial layer were poorly vascularized, suggesting that correct timing of epicardial outgrowth is essential for the normal development of the coronary network.

Although myocardial architecture seemed normal, the vascular abnormalities, seen at stage HH30, were even more severe in the embryos at HH35. Hearts of these embryos did not only display insufficient vascular penetration, but the establishment of the proximal coronary stems was also defective. As a result, the development of a proper medial layer of the coronary vessels was hampered and the coronary vessels were abnormally large and irregularly shaped.

Figure 3. Sagittal sections of a normal quail heart (HH30) (a through c) and a quail heart (HH30) with inhibition (inh) of epicardial outgrowth (d through f) stained for muscle actin (HHF35) and apoptosis (TUNEL = brown), illustrating the difference in apoptotic levels in the peritruncal region. a, Section through the aorta (Ao) where the asterisks indicate the peritruncal region. b and c, Magnifications of a consecutive section stained with TUNEL showing apoptosis in the peritruncal region. d, Section through the aorta (Ao) of heart with inhibition. e and f, Magnifications of a consecutive section stained with TUNEL showing hardly any apoptosis in the peritruncal region. LV indicates left ventricle; RA, right atrium. Scale bars = 250 μm in a and d; 50 μm in b, c, e, and f.
Earlier studies have postulated epicardial involvement in apoptosis in the outflow tract and remodeling of the outflow tract. Our study concentrates on the role of epicardium and apoptosis in coronary development. However, the incidence of outflow tract abnormalities in these embryos also supports the hypothesis that timed epicardial outgrowth mediates apoptosis in the outflow tract, which is necessary for remodeling and shortening.

To investigate how EPDCs induce apoptosis, we analyzed chimeric chicken embryos in which the quail-derived EPDCs could be traced. Because FasL/Fas-mediated apoptosis had been implicated in heart development before, we performed double immunostainings with the quail nuclear marker QCPN and with an antibody that interacts with the intracellular domain of FasL. Thus, we could visualize that specifically at sites of coronary ingrowth both EPDCs and quail-derived endothelial cells (ECs) produce FasL. By interfering with normal epicardial outgrowth, both these populations are affected and as such, also the EPDC and proepicardium-derived EC-dependent propagation of apoptotic patterning through FasL/Fas-signaling. Earlier reports demonstrated an important key-function of FasL in the region of coronary ingrowth, or at least indicate such a role. Filippatos and colleagues showed that Fas can induce apoptosis in human coronary endothelial cells. Sallee and colleagues showed that specifically the outflow tract region is susceptible to FasL. This research group also demonstrated that local hypoxia induces apoptosis in the outflow tract region. Filippatos and colleagues showed that FasL expression is upregulated by the Ets-1 transcription factor. Kavurma et al showed that FasL expression is upregulated by the Ets-1 transcription factor. Lie-Venema from our group showed that downregulation of Ets-1/2 resulted in diminished EPDC contribution to the heart and in absent coronary orifices. These results indirectly support a model in which under normal circumstances site specific hypoxia induces Ets-1 signaling in EPDCs, which causes a localized expression of FasL, contributing to coronary ingrowth.
Abnormal coronary development in animal models has been studied in relation to impaired epicardial functioning. Studies with both genetic (FOG2/H11002/H11002 in mice, antisense-Ets in chicken18) approaches, as well as with mechanical disruptions (this study) in epicardial development show a severe range in coronary malformations. This implies an important function for the epicardium in the regulation of coronary development. The malformations as seen in our study and as presented in earlier work reflect on human coronary pathology. For the purpose of understanding, we have distinguished between abnormalities of the main coronary stems and the maldevelopment of the initial intramural network.

Congenital malformations of the main coronary branches in humans have been documented and classified by Angelini.34 These include pinpoint orifices, double orifices, absence of coronary connections, and single arteries. These malformations are also seen in our avian epicardial inhibition model and presented in earlier work by Lie-Venema et al.18 an Ets-1 and Ets-2 transcription factor blocking model in chicken embryos. Gittenberger-de Groot and colleagues35 have postulated pathogenetic processes of VCACs or fistulae in humans, being a compensatory mechanism as a result of deficient coronary connections to the aorta. Fistulae were also observed in the present study, supporting the hypothesis that, when the coronary system cannot make contact with the systemic circulation through aortic orifices, the coronary vessel network searches for other ways to connect with the systemic circulation.

Our data show that apoptosis in the peritruncal ring around the aorta is not only more prominently present as compared with that in the pulmonary trunk, but the apoptotic cells are also clustered and located at specific sites were future coronary stems will develop. This implies a role for apoptosis, induced in connection with EPDCs, in establishing connections to the systemic circulation. Further investigation may reveal the intrinsic factor that causes the specifically located induction of apoptosis.

In the hearts in our study, we found a severe reduction in vascularization of the myocardium. Nevertheless, these hearts were viable, probably due to various compensatory mechanisms. However, it is imaginable that in the adult situation, when a heart functions on a fully mature vascularization, the heart has limited recruitable coronary capacity under ischemic conditions. This means that the potential for neovascularization and de novo development of collateral vessels may be impaired.

In conclusion, the presented data provide leads for future research on the epicardial contribution to myocardial vascularization. Moreover, new light has been shed on epicardially regulated apoptosis in relation to the development of the proximal coronary stems and ingrowth patterning.

Acknowledgments
This study was partly supported by grant NHS 2001B015 from the Netherlands Heart Foundation. Jan Lens is kindly acknowledged for his help in preparing the figures.
References
Coronary Artery and Orifice Development Is Associated With Proper Timing of Epicardial Outgrowth and Correlated Fas Ligand Associated Apoptosis Patterns

Ismail Eralp, Heleen Lie-Venema, Marco C. DeRuiter, Nynke M.S van den Akker, Ad J.J.C. Bogers, Monica M.T. Mentink, Robert E. Poelmann and Adriana C. Gittenberger-de Groot

_Circ Res._ published online February 10, 2005;

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2005/02/10/01.RES.0000158965.34647.4e.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/