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The epicardium as modulator of the cardiac autonomic response during early development

Tim P. Kelder [a,1], Sjoerd N. Duim [b,1], Rebecca Vicente-Steijn [a,d,e], Anna M.D. Végh [b], Boudewijn P.T. Kruithof [b], Anke M. Smits [b], Thomas C. van Bavel [a,b], Noortje A.M. Bax [c], Martin J. Schalij [d], Adriana C. Gittenberger-de Groot [a,d], Marco C. DeRuiter [a], Marie-José Goumans [b,*,1], Monique R.M. Jongbloed [a,d,**,1]

[a] Department of Anatomy & Embryology, Leiden University Medical Center, Leiden, The Netherlands
[b] Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands
[c] Department of Biomedical Engineering, Eindhoven University of Technology, The Netherlands
[d] Department of Cardiology, Leiden University Medical Center, Leiden, The Netherlands
[e] ICIN Netherlands Heart Institute, Utrecht, The Netherlands

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ABSTRACT

The cardiac autonomic nervous system (cANS) modulates heart rate, contraction force and conduction velocity. The embryonic chicken heart already responds to epinephrine prior to establishment of the cANS. The aim of this study was to define the regions of the heart that might participate in modulating the early autonomic response to epinephrine. Immunofluorescence analysis reveals expression of neural markers tubulin beta-3 chain and neural cell adhesion molecule in the epicardium during early development. In addition, expression of the β2 adrenergic receptor, the receptor for epinephrine, was found in the epicardium. Ex-ovo micro-electrode recordings in hearts with inhibition of epicardial outgrowth showed a significantly reduced response of the heart rate to epinephrine compared to control hearts. This study suggests a role for the epicardium as autonomic modulator during early cardiac development.

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1. Introduction

The cardiac autonomic nervous system (cANS) is essential in modulating cardiac function by altering heart rate (chronotropy), conduction velocity (dromotropy) and force of contraction (inotropy) [1]. Dysfunctioning of the cANS plays a role in the pathogenesis of arrhythmias [1] and hypertension [2] and is involved in disease progression in heart failure [3]. Furthermore, normal functioning of the cANS is important for the prognosis of adult patients with congenital heart disease [4]. Understanding the processes that govern normal cANS development may help in unraveling the pathophysiology of abovementioned disease processes and in developing targeted treatment options.

The cANS can be divided into a sympathetic and parasympathetic component. In general, sympathetic stimulation results in an increase of heart rate, conduction velocity and force of contraction, while parasympathetic stimulation has an opposing effect. Sympathetic neurons have their cell bodies primarily in the paravertebral stellate ganglion, whereas parasympathetic cell bodies are located in the cardiac ganglia [5]. The cells contributing to the cANS are derivatives of neural crest cells (NCCs) and cells of the nodose placode [6,7]. Kroese et al. demonstrated that prior to cardiac sympathetic innervation of the developing chicken embryo, the heart already responds to the catecholamine epinephrine [8]. This neurotransmitter binds to beta (β) adrenergic receptors (AR), thereby activating cAMP dependent signaling [9], resulting in an increase in heart rate, conduction velocity and force of contraction [5]. It is remarkable that expression of enzymes necessary for production of catecholamines was found throughout the myocardium during cardiac development in rat [10], even before production is observed in the adrenal glands [11,12]. After addition of epinephrine in chick at Hamburger and Hamilton (HH^{13}) stage 20–24, several hemodynamic parameters, including heart rate, increased significantly [8]. This

Abbreviations: β1AR, β1 adrenergic receptor; β2AR, β2 adrenergic receptor; cANS, cardiac autonomic nervous system; CTNI, cardiac Troponin I; E, embryonic day; EMT, epithelial-to-mesenchymal transition; HH, Hamburger and Hamilton; NCAM, Neural Cell Adhesion Molecule; NCC, neural crest cells; NGF, nerve growth factor; PEO, proepicardial organ; RA, retinoic acid; RXRα, retinoid X receptor-α; SV, sinus venosus; TUBB3, tubulin beta-3 chain; WT1, Wilms' tumor-1.

* Correspondence to: M.-J. Goumans, Dept. of Molecular Biology, Leiden University Medical Center, S-1-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands.
** Correspondence to: M.R.M. Jongbloed, Dept. of Anatomy & Embryology and Cardiology, Leiden University Medical Center, S-1-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands.
E-mail addresses: m.j.t.h.goumans@lumc.nl (M.-J. Goumans), m.r.m.jongbloed@lumc.nl (M.R.M. Jongbloed).
[1] Authors contributed equally.

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supports an important role for catecholamines in the heart during early embryo development. Furthermore, stimulation of chick embryos with isoproterenol (β-agonist) at embryonic day 7 (HH30–31) resulted in an increase in cAMP [14]. In addition to responding to β-adrenergic stimulation, the early embryonic chicken heart was also shown to respond to β-adrenergic receptor blockade by reducing heart rate and cardiac output [15]. Thus prior to establishment of the cANS, the heart already responds to autonomic stimulation and blockade.

Interestingly, Kroese et al. showed that after treatment with all-trans retinoic acid (RA) the reaction to epinephrine, including the increase in heart rate, was significantly reduced [8]. Normal RA signaling has been shown to be important for proper development of the epicardium [16]. This single layer of cells is derived from the proepicardial organ (PEO) and covers the initially bare primary myocardial heart tube. Cells derived from the epicardium are known to play an essential role during normal cardiac development and defects in epicardial development result in cardiac malformations (reviewed in [17]).

The aim of the current study was to identify which cell population in the developing heart plays a role in modulating the autonomic response during early development. Our studies reveal unanticipated expression of neuronal markers in the epicardium during early cardiac development. To investigate a potential role of the epicardium, electrophysiological experiments were performed with and without inhibition of epicardial outgrowth.

2. Material and methods

2.1. Animals

Immunohistochemical analysis was performed in wild type mouse embryos with a mixed genetic background of different embryonic stages (E9.5–E17.5, mice described in [18]). The morning of the vaginal plug was considered E0.5. Pregnant mice were euthanized using CO_2 exposure and cervical dislocation. Animal care was in accordance with national and institutional guidelines and approved by the animal experiments committee of the Leiden University Medical Center.

To study protein expression in chick embryos, fertilized eggs of the White Leghorn chicken were incubated at 37 °C and 80% humidity. Hearts were excised, and staged according to Hamburger and Hamilton (HH) [13]. Tissue was fixed in 4% paraformaldehyde for 24 h and subsequently embedded in paraffin and sectioned (5 μm) for immunohistochemical analysis.

2.2. Human fetal tissue

A 5-week-old human fetal heart was collected after elective abortion based on individual informed consent procedures conforming to the Declaration of Helsinki. Furthermore, the study was approved by the Medical Ethics committee of the Leiden University Medical Center. Tissue was treated as described above.

2.3. Immunohistochemistry

The protocol used for immunohistochemical staining was described previously [19]. Briefly, slides were rehydrated, subjected to heat-induced epitope retrieval and incubated with the following list of antibodies: anti-cardiac Troponin I (CTNI) (myocardial marker, 1:1000, 4T21/2, HyTest Ltd), anti-Wilms' tumor-1 (WT1) (expressed in the epicardium, 1:1000, ab89901, Abcam), anti-tubulin beta-3 chain (TUBB3) (neuronal marker, 1:200, AB78078, Abcam), Neural Cell Adhesion Molecule (NCAM) (neuronal marker, 1:250, AB5032, Merck), anti-β1 adrenergic receptor (β1AR) (receptor for epinephrine, 1:200, PA528808, Thermo Scientific), and anti-β2 adrenergic receptor (β2AR) (receptor for epinephrine, 1:200, ab61778, Abcam). To amplify WT1 expression Tyramide Signal Amplification (PerkinElmer) was used. Visualization was achieved by incubation with Alexa Fluor® 488 streptavidin (Invitrogen). The remainder of primary antibodies was visualized with Alexa-conjugated fluorescent secondary antibodies (Invitrogen) at a final concentration of 1:200. DAPI (D3571, 1/1000; Life Technologies) was used as a nuclear stain, after which slides were mounted with Prolong gold (Life Technologies).

2.4. Mechanical blocking of the proepicardial organ (PEO)

Mechanical inhibition of the epicardial outgrowth in the chicken embryo was performed as described previously [20]. At HH15, a window was created in the eggshell, after which the embryonic membranes were opened. Subsequently, a small piece of eggshell membrane was placed between the PEO and developing heart tube, after which the egg was re-incubated until the desired stage. In order to verify that outgrowth of the epicardial layer was hampered, hearts were sectioned and a Hematoxylin and Eosin staining was performed.

2.5. Ex-ovo extracellular micro-electrode recordings and epinephrine administration

To investigate the effect of epinephrine on heart rate, electrophysiological measurements were performed in embryonic chicken hearts at different developmental stages. After reaching the desired stage of development, embryos were extracted from the egg. The heart and some surrounding tissue were excised and placed in a temperature-controlled (37 ± 0.1 °C) tissue bath containing Tyrode. Recordings were performed using a previously described protocol [21]. Recording electrodes were placed on the atrium and the ventricular apex, and a reference electrode in the tissue bath. The hearts of five groups of embryos were studied: 1. HH15 embryos (n = 5), when the heart is not yet covered by epicardium; 2. HH19 embryos (n = 3), when epicardial covering of the sinus venosus, atria and AV-canal has commenced; 3. HH21 embryos (n = 3), when migration of epicardial cells around the heart is (nearly) complete [22]; 4. HH24–25 control embryos (n = 9, no surgical manipulation), when epicardial covering has been completed and subepicardial mesenchyme is present; 5. HH24–25 embryos (n = 9) after inhibition of epicardial outgrowth.

The hearts were allowed to reach a stable baseline heart rate (which was comparable between all studied groups, Supplemental Fig. S1), after which 100 μl of pre-warmed epinephrine (1 mg/ml, Centrafarm, The Netherlands) was directly pipetted onto the heart. Pre-warmed Tyrode was administered as a negative control to HH24–25 hearts (n = 6). The relative response to epinephrine was calculated by correcting the change in heart rate for the baseline heart rate. The heart rate was calculated every 10 s and plotted.

2.6. Statistical analysis

The Mann–Whitney U-test (two groups) or Kruskal–Wallis test (>two groups) were used, since the data was not normally distributed. $P < 0.05$ was considered statistically significant. Data shown is mean ± S.E.M. Statistical analysis was performed using the Graphpad Prism 6 software package (Graphpad Software).

3. Results

3.1. During early cardiac development the neuronal marker TUBB3 is expressed by the epicardium

In order to investigate which cell population plays a potential role in modulation of the early cardiac autonomic response, protein expression of the neuronal marker TUBB3 was analyzed during cardiogenesis.

At E9.5, the primary heart tube is not covered by epicardial cells and the PEO is recognizable (Fig. 1a–d). Co-expression of neuron-specific TUBB3 and WT1 was observed in a subset of cells in the PEO (Fig. 1a–d). WT1 +/TUBB3 − and WT1 −/TUBB3 + cells were also

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