Differences in development of coronary arteries and veins

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Abstract

Objective: The differentiation of the coronary vasculature was studied to establish in particular the formation of the coronary venous system.

Methods: Antibody markers were used to demonstrate endothelial, smooth muscle, and fibroblastic cells in serial sections of embryonic quail hearts. The anti-β-myosin heavy chain and the neuronal marker HNK-1 were added to our incubation protocol.

Results: In HH32, the coronary vascular network has developed into a circulatory system with connections to the sinus venosus, the aorta and the right atrium. The connections between the aorta and the right atrium allow for direct arteriovenous shunting. Subsequently, differentiation into coronary arteries and veins occurs with an interposed capillary network. The smooth muscle cells of the coronary arterial media derive from the subepicardial layer, whereas the subepicardially located cardiac veins recruit atrial myocardium, as these cells express the β-myosin heavy chain antigen. Ganglia are located in the subepicardium close to the vessels, while nerve fibres tend to colocalize with the formed vessel channels.

Conclusions: A new finding is presented in which the subepicardial coronary veins have a media that consists of myocardial cells. The close positional relationship of neural tissue and coronary vessels that penetrate the heart wall is explained as inductive for vessel wall differentiation, but not for invasion into the heart.

Keywords: Heart development; Coronary vasculature; HNK-1; β-Myosin heavy chain; Quail; Cardiac autonomic nervous system

1. Introduction

The development of the coronary vascular system and its differentiation into the main coronary arteries has been documented for the human [1–3], as well as for the avian embryo [4,5]. However, knowledge concerning the differentiation of coronary vessels and in particular the coronary venous system is still inconclusive.

The initial development of the endothelium-lined coronary plexus has been reported by our group [6,7]. Also results on the formation of a media around the main stems of the coronary arteries using the anti-actin antibody, HHF35 [5], and the 1E12 antibody [7] have been reported. In addition, an adventitial layer appears around the coronary arteries, as shown with the anti-procollagen-I antibody, M38 [7].

The subject of our present study is to single out the formation of coronary veins from the development of the complete coronary vasculature in the embryonic quail heart. We have investigated the development of the coronary venous system from HH30 onward, using the QH1 antibody [8].

In contrast, the wall of the coronary veins did not stain with the smooth muscle cell marker, 1E12. Therefore, we incubated consecutive sections of quail hearts with an anti-myosin heavy chain (MHC) antibody against chicken atrial cells [9], to explore the contribution of atrial cells to the media of the coronary veins.

The HNK-1 antibody has been used to examine the formation of the cardiac neural plexus, as this antibody is a
marker for migrating neural crest cells and also for the developing peripheral nervous system in the avian embryo [10]. HNK-1 can be used as a marker for neural crest cells in the avian embryo only to some extent, because non-neural crest derived structures such as the wall of the sinus venosus and the ventricular myocardium may also be labeled [11]. A relationship between the appearance of cardiac ganglia within the subepicardial layer of the heart and the presence of coronary arteries within their vicinity has been documented [12,13]. We have used the HNK-1 monoclonal antibody to examine a possible correlation between the presence of cardiac neural tissue and the formation of the coronary venous system in the quail heart.

This study investigates the development of the coronary vessel plexus, as it differentiates into the main stems of the coronary arteries and veins. We also attempt to define the origin of the cells of the medial layer around the endothelium of the coronary vessels. In addition, we studied the correlation between the presence of the neural crest derived neuronal cells and the site of penetration of coronary vessels into the aorta [4,6] and right atrium [7].

2. Methods

2.1. Preparation of the embryos

Japanese quail embryos (Coturnix coturnix japonica) were staged according to Hamilton and Hamburger for chicken embryos [14]. Embryos ranging from HH30 to HH44 (6.5 to 15 days) were used. After incubation at 37.5°C (80% humidity), the quail embryos were carefully removed from the yolk and transferred to a small dish with Locke’s salt solution (0.94% NaCl, 0.045% KCl and 0.004% CaCl₂). The thorax segment (HH30 to HH38) or only the heart-lung-specimen (from HH38 onward) were fixed in 2% acetic acid in 100% ethanol at 4°C, for at least 48 h. After embedding in paraffin, the tissue was serially sectioned transversally or sagitally at 5 μm and the sections were transferred to albumin/glycerin coated objective slides.

2.2. Immunohistochemistry on serial sections

The deparaffinized and rehydrated sections were immersed in phosphate-buffered saline (PBS), pH 7.4, to which was added 0.3% H₂O₂ to inhibit endogenous peroxidase activity. After rinsing twice in PBS for 10 min and once in PBS with 0.05% Tween-20 (PBS-Tween, Sigma) for 10 min, overnight incubation at room temperature took place with the first antibody. Consecutive sections were incubated with either the 1:500 diluted QH1 antibody [8], the (1:1000) HHF35 antibody [15], the (1:10) HNK-1 antibody [16], the (supernatant, 1:10) 1E12 antibody [17], the (1:3) M38 antibody [18], or with the (1:15) anti-β MHC antibody (code 169-ID5) [9]. The antibodies were diluted in 1% ovalbumin in PBS-Tween-20. After rinsing, the second incubation was performed for at least 1 h with the (1:200) rabbit anti-mouse peroxidase conjugated antibody (Dakopatts P260, Glostrup, Denmark). The 1E12, β-MHC and M38 antibody-incubated sections were rinsed again and incubated for at least 1 h with a third antibody, the (1:50) goat-anti-rabbit immunoglobulin (Nordic, Tilburg, The Netherlands), rinsed and incubated for one hour with a fourth antibody, the (1:500) rabbit peroxidase-anti-peroxidase antibody (Nordic). Subsequently, all sections were rinsed twice in PBS, 10 min, and once in 0.05 M TRIS-maleic acid (TRIS-mal), pH 7.6, 10 min. The peroxidase staining reaction was performed by exposing the slides 8 min to diaminobenzidin (Sigma) diluted in TRIS-mal, to which was added 0.006% H₂O₂, followed by washing in buffer. The sections were briefly counterstained with Mayer’s hematoxylin, dehydrated in ethanol, covered with Entellan (Merck, Darmstadt, Germany), coverslipped and investigated by light microscopy.

2.3. Specificity of the primary antibodies

The HHF35 antibody recognizes skeletal, cardiac, and smooth muscle alpha actin and smooth muscle gamma actin [15]. The 1E12 antibody labels embryonic and adult smooth muscle cells of the chick, but not the developing cardiac muscle. Characterization of the 1E12 antigen points towards α-actin [17]. The quail specific QH1 antibody reacts with endothelial cells lining a vessel lumen, and cells that belong to the hemopoietic cell lineage. These cells can be distinguished from one another on the basis of time of appearance and location within the embryo [8]. Although the QH1 epitope is unknown, it is commonly used as a marker for endothelium. In the present study we used a monoclonal antibody directed against the beta isoform of the MHC molecule, recognizing both the non-specialized atrial myocytes and the Purkinje cells of the conduction system of the heart [9]. The HNK-1 antibody reacts with glycoproteins and glycolipids of e.g. the central and peripheral nervous tissue [16].

3. Results

3.1. The formation of the coronary vascular system

In HH30, all the coronary vessels are endothelial-lined tubes. The vascular network is connected to the sinus venosus on the dorsal side of the heart (Fig. 1A) from which it receives its blood. These connections will develop into the future coronary veins, connected to the coronary sinus. At the ventriculo-arterial transition, a peritruncal ring of vessels is present around the great arteries. In HH32, two lumenized connections appear between the peritruncal ring and the two semilunar sinuses of the aorta facing the pulmonary artery. These two connecting vessels
are the first Anlage of the proximal stems of the coronary arteries (Fig. 1B). From this stage onward the network will be supplied through the aorta. Other lumenized connections appear between the peritruncal ring and the ventral aspect of the right atrium. These vessels are the first Anlage of the proximal stems of those coronary veins that

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Fig. 1. Serial sagittal sections of embryonic quail hearts of consecutive stages, namely HH31 (A) and HH32 (B,C), stained with the QH1 antibody. (A) The endothelium-lined coronary vessels (C) in the subepicardium (E) of the dorsal interventricular groove and in the ventricular myocardium (M) are in open connection with the sinus venosus (SV). (B,C) Show the arteriovenous shunt via the peritruncal ring. The two micrographs are of the same embryo in which (C) is located 70 \( \mu \)m more to the right lateral side of the heart than (B). (B) A proximal coronary artery (CA) connects the coronary vessels (C) of the peritruncal ring with the aorta (AO). (C) A proximal coronary vein (CV) connects the coronary vessels (C) of the peritruncal ring with the right atrium (RA). LV left ventricle; RV right ventricle.
Fig. 2. Sagittal sections of consecutive stages of quail embryos, namely HH32 (A), HH36 (B), HH38 (C), and HH40 (D) in which the differentiation of coronary vessels into arteries is illustrated. (A) A consecutive section of Fig. 1B is stained with 1E12. An area corresponding to the boxed area of Fig. 1B is enlarged in panel A. The first smooth muscle cells are visible (arrowheads), lying close to the coronary artery endothelium and the aortic media (ME). (B) A media is present around a coronary artery (CA) in the interventricular septum (IS). (C) A small area of the aortic sinus (S) where the coronary artery invades this structure (arrowheads) remains 1E12-negative at all time during coronary artery differentiation. (D) Pro-collagen type I-positive dots (arrowheads) are lying within the adventitia around the coronary arteries (CA) and coronary veins (CV). The inset shows the same section in which the background is mitigated by a blue filter for a better distinction of the positive dots. Note the adjoining localization of these two coronary vessels in a combined vessel channel. Only within the subepicardium do the coronary veins attain a three-layered structure at this developmental stage. Formation of venous media or adventitia is never observed extending into the ventricular myocardium. AO aorta; E subepicardium; M myocardium; RV right ventricle.
have a separate connection to the right atrium (Fig. 1C). Thus, in HH32, the vascular network is supplied by the aorta and is drained via the sinus venosus and right atrium, allowing for direct arteriovenous shunting via the peritruncal ring. Lumenized connections between the vascular plexus and the left atrium have not been encountered.

Fig. 3. Sections of different stages of quail embryos, namely HH43 (A), HH38 (B), HH42 (C), and HH40 (D) in which the differentiation of coronary vessels into veins is illustrated. (A) The micrograph of an anti-actin stained transversal section through the arterial orifice level shows the existence of a coronary artery (CA) and a coronary vein (CV) in a joined vessel channel. (B,C) Sagittal sections through the proximal coronary veins (CV) connected with the sinus venosus (SV) (B) and with the right atrium (RA) (C) reveal that the media is comprised of β-MHC-positive cells (arrowheads). (D) A sagittal section through a more distally located coronary vein (CV) reveals that the media is comprised of 1E12-positive-cells (arrowheads). AO aorta; E subepicardium; M myocardium; PA pulmonary artery.
3.2. Immunohistochemistry of the media and adventitia

After the vascular network has contacted the aortic lumen at HH32, the first 1E12-positive mesenchymal cells can be noted close to the endothelium of the most proximal coronary artery stems adjacent to the aortic media (Fig. 2A). These cells are the first components of the artery medial wall. The media increases both in thickness and in length along the arteries, located in the myocardium of the interventricular septum and in the subepicardial layer of the atrio-ventricular groove (Fig. 2B). A small part of the wall of the aortic sinus, just surrounding the coronary orifice remains 1E12-negative (Fig. 2C). From HH32 onward, pro-collagen-I producing fibroblasts, demonstrated with M38, appear within the adventitia of the proximal artery stems. The extension of adventitia formation (Fig. 2D) occurs in concert with the arterial media formation.

A common feature observed during vascular maturation, is the existence of an artery and a vein in a double vessel channel (Fig. 3A). These channels have invaded deep into the myocardium and nearly encircle the atrio-ventricular groove. At HH32, the veins still lack a media and adventitia. In HH39, actin-expression becomes apparent in cells adjacent to the endothelium of the most proximal part of the veins located within the subepicardial layer of the sinus venosus and the right atrium. These actin-positive cells also express the β-MHC antigen, as found in the atrial cardiomyocytes (Fig. 3B,C). The extension of this atrial sleeve proceeds from both the right...
atrium and sinus venosus along the ventral and dorsal coronary veins towards the ventricular myocardial boundary, where the veins become embedded in the ventricular mass. At HH43, we have noted that more peripherally located cells within the venous media also stain positively for the 1E12-antibody (Fig. 3D). The extension of a venous adventitia develops in concert with the venous media. While smooth muscle cells and fibroblasts adjoin the arteries deep into the interventricular septum, the veins that colocalize within the myocardial vessel channel never develop a three-layered structure. Their endothelium still adjoins the ventricular myocardial cells until late stages of coronary vascular development.

3.3. The location of HNK-1 positive cells in relation to the formation of coronary arteries and coronary veins

In HH30, HNK-1 positive cells have invaded the dorsal mesocardium to reach the sinus venosus. These cells form conglomerates of ganglia located in the subepicardium adjacent to the vascular plexus connected to the venous side of the heart. HNK-1 positive neuronal fibres run from these ganglia over the dorsal side of the heart in close relation to the expanding vessels (Fig. 4A).

At HH31, HNK-1 positive cells around the outflow tract of the embryonic heart have invaded the level of the peritruncal ring. These ganglia are mainly situated around the aorta, in the septum between the aorta and the pulmonary trunk and between the aorta and the right atrium (Fig. 4B). They colocalize with the sites where the coronary arteries will invade the aorta and the coronary veins the right atrium (Fig. 4C).

From HH32 onward, nerve fibres migrate from the outflow tract ganglia to invade the myocardium of the interventricular septum and the subepicardium of the atrio-ventricular groove. Migration of nerve fibres occurs in the developing vessel channels in the direction of the apex of the heart. A close relation between the migrating nerve fibres and the formation of a media around the coronary arteries of the vessel channel can be detected (Fig. 4D). At HH42, nerve fibres are not only limited to the developing main vessel channels, but they become widely scattered throughout the myocardial and subepicardial layer (Fig. 4D).

3.4. The extension of atrial myocardium in the media of the venous inflow of the heart

The proximal part of the media of the great veins in the body wall is also comprised of atrial myocardium, comparable to coronary venous media formation. From HH38 onward, the myocardial cells, detected with the β-MHC antibody, extend from the left atrium around the pulmonary venous endothelium (Fig. 5A), and from the right atrium around the caval venous endothelium (Fig. 5B).

4. Discussion

Extensive research has been performed on the growth and differentiation of the coronary vascular system in avian embryos using histological [19,20], immunohistochemical [4,5,21] and experimental techniques to elucidate the origin and development of the coronary arteries [5,6,12,22–24]. However, the formation of the coronary venous system and the origin of its components is still unrefined.
An important source of cells, the proepicardial organ, is located between the sinus venosus and the primordial liver and its protruding villi reach towards the dorsal surface of the embryonic heart and contact the myocardial surface at about HH16 [25]. The epicardial sheath derives from the proepicardium [26–28]. Moreover, it encompasses the entire endothelial lining of both the coronary arterial and venous system [6], whereas the progenitor cells of the smooth muscle cells in the media and the fibroblasts in the adventitia of the arteries are of proepicardial origin [23].

Although, the number of layers of which both arteries and veins are comprised is the same, differences can be noted in the timing of vessel wall differentiation, the origin of media cells, and the extent of the media in the myocardium.

The arteries have started to differentiate at HH32, whereas the veins will not attain a media or an adventitia before HH39, which is well after the time that the vessels of the peritruncal ring have contacted the right atrium.

The media of the veins contains other cell types than the arteries. It stains with anti-actin, whereas the smooth muscle cell marker for the arteries, 1E12, did not provide a staining of the proximal part of the venous media. Here, we showed expression of the β isoform of the atrial specific myosin heavy chain. The media of the proximal part of the veins derives from the myocytes of the atrial wall. More distally the media became mixed and some cells that were negative for the myocardial marker were positive for 1E12. Although speculative, this variation in phenotype could reflect the various different vasomotor responses that these cells show to transmitters [29].

The extension of cardiac musculature as a tunica media sleeve around the pulmonary and caval veins has been noted before in mammals [30–33]. However, this phenomenon has not been described for coronary veins. We conclude that the atrial musculature surrounds the complete venous pole of the heart. We expect that the coronary venous myocardial cuff will play a role in preventing backflow of blood from the right atrium during atrial contraction.

Another important difference between arterial and venous wall development, is the extent of the venous media and adventitia limited to the subepicardium, never entering the myocardial wall. The endothelium of the veins within the interventricular septum closely adjoins the ventricular myocytes without having a differentiated vessel wall. This is observed up to hatching in the quail. Probably, the intramyocardial veins lack vasoactive activity, while the venous blood is probably squeezed into the proximal veins due to ventricular contraction.

At about HH15-HH19, neuronal parasympathetic ganglia, most likely originating from the cardiac neural crest, invade the sinus venosus region by way of the dorsal mesocardium [11,13]. This is the period in which the Anlage of the coronary vasculature becomes apparent [6,7]. The nerve fibres, that partly derive from the sympathetic crest in later stages, colocalize with the undifferentiated vessels in the dorsal subepicardium. Spence and Poole [34] stated that the formation of blood vessels preceded the migration of neural crest cells that use the developing blood vessels as a substratum.

Large ganglia are found in the subepicardium of the outflow tract at HH30, when the vessels of the peritruncal ring are not yet connected to aorta or right atrium. As in the next stage of development, ganglia are found in areas of connection with the aorta and right atrium, as well as near the aortic non-facing sinus, pulmonary trunk and left atrium, lacking connections with the peritruncal ring, we conclude that nerve fibres apparently do not induce endothelial penetration.

The presence of ganglia in accordance with media formation of the arteries has been reported earlier [5] and is supposed to be essential to the survival of the definitive coronary arteries [6,12,13]. Although the association of ganglia with persisting coronary arteries may suggest that chemotactic substances released by neural tissue is in some way essential to the development of the walls of the arteries, it can not be the only determining factor. In a combined vessel channel nerve fibres are as closely related to the veins as to the arteries. The veins also develop a medial layer, however, in a different time schedule, which seems to rule out neuronal influence. Experimental ablation of neural crest does not prohibit vascular smooth muscle cell deployment, as coronary arteries still contain a medial layer [5]. The presence of neuronal ganglia not related to proximal coronary arteries in an ablation model of persistent truncus arteriosus shows the complexity of this matter [13]. Substances released by endothelial cells can facilitate vascular smooth muscle recruitment [35,36]. As increased shear stress can induce this release [37], we suppose that the increase in pressure and alteration in blood flow after connecting to the aorta, is a potential factor involved in smooth muscle differentiation around the arterial endothelium. This phenomenon may explain the relative late differentiation of the venous wall in the low pressure environment of the coronary veins.

Several questions are left to be elucidated. We are inquisitive about the origin of the vascular smooth muscle cells around the veins that do not express the β myosin heavy chain but stained positive for the anti-actin antibody, HHF35, and the smooth muscle marker, 1E12. We speculate that these cells are also derived from the same extracardiac source that gives rise to the media of the arteries [23]. We are presently transferring liver-proepicardium pieces of the quail to the chick pericardial cavity to answer this problem.

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References


