University of Medicine and Pharmacy
Craiova

PhD THESIS

CONTRIBUTIONS TO THE HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF HUMAN MYOCARDIAL DEVELOPMENT AND ITS VASCULARIZATION

- ABSTRACT -

SCIENTIFIC SUPERVISOR:
Prof. Univ. Dr. Laurențiu MOGOANTĂ

PhD STUDENT:
Ionel Dorin MARINAȘ

CRAIOVA

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INTRODUCTION

The heart is the first functional organ that occurs in the developing embryo. Many studies have been done on different animal models in order to observe the time frames for the formation of mature functional myocardocytes. For example, in chick embryos (which fully develop in 21 days), contractions of cardiomyocytes can be observed after only 36 hours from the fecundation, and blood flow through the heart begins at 2 days after conception. Sparsely distributed myofibrils begin to be visible in the myocardocytes at around 30 hours post-fecundation, then the first Ca2+ - Mg2+ ATP-ases become active, and around the 1.5 days stage the first action potentials occur and precede the sustained contractions. Cultured myocardocytes have been also studied for the dynamics of fibrillar and pre-fibrillar assemblies of actin and myosin filaments.

Despite this, not much is known regarding the actual stage of sarcomere formation in human heart in vivo. At the age of 3-4 weeks, the human heart starts to contract, but to what extent the sarcomeres are structurally developed it is not really know.

Intermediate filament cytoskeletal proteins (IF) have important functional and structural roles in any tissue, even from embryonic stages. Desmin forms a scaffold for future contractile filaments in both cardiac and skeletal striated muscle cells, bridging the edges of Z lines together, and maintaining their connection with mitochondria, nuclei, and sarcolemma. Vimentin on the other hand, is found in fibroblasts and other cell types, close to the cytoplasmic surface of membrane-to-membrane contact points between cells, maintaining the stability of mesenchymal cells and their precursors.

Besides other cytoskeletal proteins, actin is a fundamental protein supporting the contractile apparatus in muscle cells. In mammals, the muscle actin family is composed of six distinct isoforms. Out of these isoforms, the β-cytoplasmic and γ-cytoplasmic actins are ubiquitous cytoplasmic proteins, while α-skeletal actin (α-SKA), α-cardiac actin (α-CAA), α-smooth muscle actin (α-SMA) and γ-smooth muscle actin (γ-SMA) are found in muscle tissue. In normal myocardium, α-CAA, α-SKA and α-SMA isoforms are co-expressed at levels depending on the species, developmental stage, or pathological alterations. Experiments on animal models showed that during heart formation, α-SMA is expressed by cardiomyocytes during their differentiation, and during later moments in development it is replaced by α-SKA and α-CAA. Although the importance of this phenomenon is not known, the study of α-SMA dynamics on human heart for different stages has not yet been done.

Vascular development and maturation is an important step in the development of any organ, and especially in such high energy-dependent organs like the heart. CD31 (PECAM-1), is a single chain type I transmembrane protein, which mediates adhesive interactions between adjacent endothelial cells as well as between leucocytes and endothelial cells. Although studies on developing mammalian and bird cardiovascular system had been done using lectins and antibodies raised against factor VIII in order to identify endothelial cells, these detected mostly carbohydrate residues present on mature endothelial cells, but not so much on developing endothelial cells. Data in the literature shows that PECAM-1 is one of the earliest adhesion molecules expressed by developing endothelial cells, but an extensive study in human developing heart was not reported yet. Endoglin (CD105) is a transmembrane glycoprotein, mainly expressed by the endothelial cells in newly formed vessels in the state of active angiogenesis, and absent in most other vessels in mature tissues [16]. Endoglin has been also shown to play an important role in development of the vascular and cardiac system. For example, heterozygous for deleterious mutations in the endoglin gene are known to led to the occurrence of vascular malformations.

The aim of this study was to attempt to describe the localization of both desmin and vimentin in the developing human heart between 5 and 33 weeks of gestation, in combination with the above two mentioned markers for vasculature and actin.
Keywords: miofibrilogenesis, smooth muscle actin, desmin, vimentin, angiogenesis, heart development.

I. GENERAL PRESENTATION

Throughout two chapters, Chapter I – Heart ontogenesis and Chapter II – Heart anatomy and histology, in the light of the bibliographic data, it is widely described the heart development and and its macroscopical and microscopical structure.

II. OWN CONTRIBUTIONS

Objectives of the study:
- describing the different stages of human heart development and its blood vessels in classical histological stain with standard Harris hematoxylin and a modified Heidenhain’s iron hematoxylin.
- the age of appearance of the first cardiomyocytes’ striations in light microscopy and their initial location.
- the coherence between appearance of first sarcomeres and nuclear mitotic capacity of human cardiomyocytes, knowing from studies on animals that in the second part of gestation and in perinatal period the majority of cardiomyocytes had lost their proliferative capacity developing mainly by hypertrophy.
- examination of the heart’s vessels development analysing the expression of CD31 and CD105 at the level of vascular endothelium and growing endocardium.
- showing the implication of α-smooth muscle actin in the building and organisation of sarcomeres of the human cardiomyocytes, given that there are no studies on human embryos in this field.
- analysing the location and function of desmin and vimentin throughout human fetal heart development.

Chapter III entitled “Material and methods” describes the study materials, as well as used techniques.

All the human embryos needed for this study were collected following miscarriages in the gynecology department of the Emergency County Hospital 1, Craiova (Table 3.1). The gestational ages were calculated as the time passed from the last menstruation till the moment of harvesting the tissue.

The whole embryos were fixed in 10% neutral buffered formalin for 4 days, and then were dissected and the hearts isolated and routinely processed for paraffin embedding (department of Histology, University of Medicine and Pharmacy Craiova). Serial sections were cut on a rotary microtome (Microm), and collected on poly-lysine-coated slides. Macroscopical and histological analysis showed that the embryos selected for our study did not have heart malformations.

**Table 3.1. Number and ages of the embryos' hearts for this study**

<table>
<thead>
<tr>
<th>Embryo's age (weeks)</th>
<th>&lt;5</th>
<th>5</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>20</th>
<th>33</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of hearts available</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
From the pieces included for paraffin seriated sections were cut with a thickness of 4 μm. **Histochemically, classical staining** procedures were used, such as haematoxylin-eosin and modified Heidenhain’s iron hematoxylin.

For the **immunohistochemical study**, the same biological material was used as for the histological investigations. The imuno-histochemical technique per se included a standard algorithm, with some variations depending on the used antibodies (Table 3.2).

The two dimensional automated morphometry was aimed at:
- Semi-quantitative measurement of PCNA-positive nuclei as the percentage from the total counted nuclei in different stages of myocardial development.

### Tabel 3.2. Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope / marker</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD105</td>
<td>Proliferating vessels</td>
<td>1:100</td>
<td>Citrate, pH=6</td>
<td>Labvision</td>
</tr>
<tr>
<td>CD31</td>
<td>Vascular endothelium</td>
<td>1:50</td>
<td>Citrate, pH=6</td>
<td>Dako</td>
</tr>
<tr>
<td>Desmin</td>
<td>Muscle cells</td>
<td>1:50</td>
<td>Citrate, pH=6</td>
<td>Dako</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Mesenchymal cells</td>
<td>1:100</td>
<td>Citrate, pH=6</td>
<td>Dako</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cells</td>
<td>1:100</td>
<td>Citrate, pH=6</td>
<td>Dako</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Smooth muscle actin</td>
<td>1:100</td>
<td>Citrate, pH=6</td>
<td>Labvision</td>
</tr>
</tbody>
</table>

**Chapter IV – Results and discussions**

**4.1. Histological study results**

On the youngest developing heart in the study, histology investigation identified mesenchymal cells covering thorough the cardiac vesicle areas. These cells were mostly stelate, with ramified arms joining together with the neighbouring cells to form a cellular net.

Beginning with 3-4 weeks embryo heart, the cells began to look more distinct, with enlarged and clear cytoplasm, the nucleus being most frequently round, hypochromatic and centrally situated. The same aspect was constant throughout the week 14, with the ventricle areas having denser cells compared to the future atrial areas. Beginning with this age, the cells began to have a more elongated shape as mature cardiomyocytes.

At 33 weeks, the cardiomyocytes had a mature striated pattern, some of them being binucleated. We also found at this age vegetative ganglia in atrial epicardium.

On Heidenhain’s haematoxylin, in the week 15, the first sarcomeres began to be visible, probably reflecting a previous state of myofibril existence and aggregation, and now were dense enough to be visible on light microscopy. In all three embryo hearts available for this gestational age, the first light microscopy visible sarcomeres appeared at the edge of the cell, just beneath the plasma membrane. This arrangement was constant all around the membrane, with no clear interruption, and constant again for ventricular and atrial areas.

**4.2. Immunohistological study results**

**4.2.1. PCNA expression**

On PCNA immunohistochemistry, as expected, we noted a decreasing nuclear staining with increasing embryonic age. In very young hearts (<5 weeks), practically all mesenchymal cells’ nuclei strongly expressed PCNA. At this age we counted around 25,4% (±8,1%) positive
nuclei. The nuclear positivity decreased with a relative constant slope at 21.5% (±7.6%) for 12 weeks and to 18.3% (±11.7%) for 15 weeks.

After 16 weeks of age however, the nuclear positivity dropped-out drastically at 3.3% (±1.6%) at 17 weeks, and respectively at 1.8% (±1.8%) for 20 weeks of age. After this age there was an almost constant PCNA staining percentage in myocardocytes.

Paralleling the formation of the first light microscopy visible sarcomeres and the described drop-out in PCNA-nuclear positivity, we observed that the two phenomenon did not coincide and in all studies cases of respective ages, formation of the first visible sarcomeres was followed by a two week interval after which the nuclear maturation drastically increased (as revealed by lower PCNA-staining).

4.2.2. CD105 and CD31 expression

Immunohistochemistry for endoglin showed that the reactivity begins to occur in human heart at around 5 weeks, in the future endocardial layer. While at this age there were no positive vessels in the myocardium itself, immunoreactivity begins to occur in endothelial cells during the weeks 9-10. After this, the signal could be strongly detected in endocardial, myocardial and epicardial vessels.

Already at 5 weeks of development, anti-CD31 immunostaining detected scattered fully stained endothelial cells and vessels in the myocardium, while the endocardial layer was not yet fully expressing this marker. By 7 weeks, more vessels could be noted, and endocardial layer was expressing CD31 on its complete lining. At the embryo age of 10 weeks, large vessels are observed in the epicardial layer. The number of vessels was relatively constant till around the age of 20 weeks when their density increased rapidly. Overall, there was an increasing expression of both CD105 and CD31 with age of the embryo, mature vessels seeming to retain an endoglin positive phenotype.

4.2.3. Desmin and vimentin expression

Analysis of the slides immunostained for desmin, showed that reactivity begun to appear at the age of 9 weeks, with increasing intensity and area staining from 10 weeks onward.

Desmin was present only in cardiomyocytes. At stages of initial detection point, desmin seemed to be lining the cardiomyocyte plasma membrane. The staining pattern seemed to be stronger in the immediately sub-epicardial layer compared to myocardium itself. Beginning with 17 weeks of gestational age, desmin was also localized in the myofibrile striations in the cytoplasm of the cardiomyocyte. This feature reached the pattern of the mature striated muscle cell beginning with 17 weeks of age onwards. At 33 weeks appeared the first binucleated myocytes and intercalated discs.

Vimentin was already present in the mesenchymal cells from the first investigated time point. As the blood vessels lumens become more clearly visible, vimentin showed also a perivascular localization. Vimentin was heavily expressed in aorta smooth muscle cells, while desmin could not be detected in this compartment. Endocardium lining at the level of future atrio-ventricular valves was also positive for vimentin. With increasing age of the embryo, vimentin expression diminished in the myocardium itself, remaining present around the vessels and to a higher extent in the sub-epicardial vessels.

4.2.4. α-SMA/CD31 expression by immunofluorescence

After composing the α-SMA/CD31 double images, in the non-vascular compartment, we also observed a gradual variation of α-SMA expression during different developmental stages. Thus, except a slight tissue autofluorescence, no mesenchymal cells were α-SMA-positive below the age of 7 weeks. After this age, α-SMA began to be expressed in the future myocardium cells, showing a sub-membranous expression pattern somehow related with the expression of desmin presented above. Examining these patterns, on week 15 we observed for the first time the formation of striat-like sarcomeric structures, suggesting a role for α-SMA in temporary
scaffolding the myofibrils in cardiac striated muscle. In the later weeks, SMA expression revealed the typically striated structure of the sarcomeres, but with an abrupt decrease between the 20th and the 33rd weeks of development. Finally, after 33 weeks, no α-SMA could be detected in the cardiomyocytes’ compartment, like in the post-partum and adult heart tissue (data not shown). On parallel, forming endothelial cells show a dotted express of CD31, besides individual and well defined vessels. Beginning with 20 weeks, CD31 expression is clearly present only as clear-cut vessel walls.

In the vascular compartment, CD31 was first detected both with a granular appearance in endothelial cells, some of them not having yet clear-cut lumens; but also in different sized vessels, some of them large enough to pose a proper sheet of smooth muscle cells. Beginning with 17 weeks of age, larger vessels clearly presented an α-SMA positive tunica media and an independent and non-granular endothelial layer.

**CONCLUSIONS**

- Our study showed that myofibril formation in human cardiomyocytes begins to be visible on light microscopy at embryonic age of 15 weeks, beneath the cellular membrane. This parallels in vitro or animal model studies describing the dense plaques that seem to initiate myofibril formation and which seem to be anchored on the inner side of the plasma membrane.

- We have found a sudden nuclear maturation between weeks 15th and 17th, with a rapid loss of nuclear cell division protein PCNA. Interestingly, this maturation phenomenon occurred after the fist myofibrils begun to be observed on light microscopy. Given the fact that electron microscopy might in fact detect these structures earlier in their evolution, this time gap is even larger than 2 weeks. Thus it seems that myocardocytes retain their mitotic capacity until after they begin myofibril synthesis.

- Our study compared for the first time the expressions of CD31 and CD105 in the analysis of heart vasculature development, together with three wide distributed cytoskeletal filaments.

- We showed that there are not only time-point differences in the expression of these markers, but also structure-related differences. Thus we show that while CD105 occurs in the endocardium, CD31 is present much earlier in the future myocardium area and that angiogenesis begins around 10 weeks.

- Regarding the cytoskeletal elements, α-SMA and desmin seem to be close related to myofibril formation as they both start at the periphery of the myocyte, while vimentin seems to be mainly driving the integrity of the supporting tissue.

- Vimentin was also found inside aortic wall and at the level of future atrioventricular valves. Along with embryo’s growth, vimentin expression decrease in myocardium itself, remaining present around vessels and especially in subepicardial vessels.

- Immunofluorescence study described for the first time transient occurrence of α-SMA in human fetal heart cardiomyocytes between 9 and 20 weeks of development, with its dissapareance from myocardium before week 33, suggesting a function in forming the sarcomeres. Beginning with week 33, α-SMA expression was present only in tunica media of vessels.
We noticed the appearance of first binucleated myocytes and intercalated discs at 33 weeks. At this age we also found well-structured vegetative ganglia in the atrial epicardium.

As the dynamics of heart formation and disease is driven by the balance between the vascular, myocytic and stromal components, understanding their interactions will improve our knowledge in both directions.

CURRICULUM VITAE

NAME
Ionel Dorin MARINAȘ

BIRTH DATE
January 25th, 1980, Corabia, OLT County

CURRENT AFFILIATION
Emergency County Hospital Craiova, Cardiology Center, – resident cardiologist

STUDY:
1995 - 1999: Alexandru Ioan Cuza College of Corabia, Olt;
1999 - 2005: Medicine Faculty from University of Medicine and Pharmacy Craiova.
2006 - 2008: Resident doctor in Pneumology
2009 - present: Resident doctor in Cardiology

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