Rate of coronary vascularization during embryonic chicken development is influenced by the rate of myocardial growth

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Abstract

Objective: We tested the hypothesis that the degree of coronary microvessel formation in the embryonic heart is regulated by the magnitude of myocardial growth. Methods: The outflow tract of Hamburger–Hamilton stage 21 chicken hearts (prior to the onset of coronary vasculogenesis) was constricted in ovo with a loop of 10-0-nylon suture, and the hearts were studied at stages 29 and 36. Results: At stage 29 ventricular mass was 64% greater in the pressure-overloaded than in the hearts of sham-operated controls, but vascular volume density and numerical density, determined by electron microscopic morphometry, were identical. As demonstrated by histological morphometric evaluation, the compact region of the left ventricle at stage 29 was 43% thicker than the shams. However, by stage 36 heart mass, thickness of the compact region, and overall wall thickness (demonstrated by scanning electron microscopy) were significantly less than in the sham group of this stage, but vascular volume density was virtually identical in the two groups. Formation of the two main coronary arteries was clearly impeded in the banded hearts, i.e., the coronaries were stunted in their development or failed to completely form coronary ostia. Conclusions: Vascular growth is proportional to myocardial growth in the embryonic, overloaded heart, but the persistence of the pressure overload results in a failure of or severe limitations in coronary artery development. These data support the hypothesis that vascular growth during this period of development is regulated, at least in part, by the rate and magnitude of myocardial growth.

Keywords: Pressure overload; Chicken; Electron microscopy; Angiogenesis; Vasculogenesis

1. Introduction

The vascular response to cardiac enlargement in the adult is variable and dependent upon several factors including the stimulus evoking the increase in ventricular mass, age, and the duration of hypertrophy [1,2]. Coronary angiogenesis (sprouting of vessels from pre-existing vessels) may be stimulated by mechanical factors such as enhanced flow and shear stress, or stretch resulting from increased ventricular filling [3,4]. During early normal postnatal growth a marked capillary proliferation occurs, and then is attenuated as capillary growth does not keep pace with myocyte growth, and capillary density becomes stabilized to adult values [5]. Since vasculogenesis (vessel formation by endothelial cell precursors) constitutes the initial phase of neovascularization in the embryo, comparisons to postnatal growth are not entirely appropriate. Moreover, little is known about the role of ventricular mass in determining vascular parameters in the embryo because coronary vascular growth during the prenatal period has not been as extensively studied.

We previously demonstrated that when ventricular pressure is increased in the chicken embryo the consequential increase in cardiac mass is due to hyperplasia rather than hypertrophy of myocytes [6]. In our model, the conotruncus is constricted with a loop of 10-0 nylon suture at Hamburger–Hamilton (HH) stage 21, which results in a...
substantial increase in the ventricular dry weights of the constricted hearts compared to sham controls at stage 29. Thus, the heart is able to adjust to loading conditions by increasing its mass even during the embryonic period. Since this period of development is characterized by the onset of vasculogenesis (at approximately stage 22–23) the increase in cardiac mass occurs during myocardial vascularization. In humans, congenital cardiac enlargement due to pressure overload is characterized by normal vascular densities, while cardiac enlargement acquired in adults is not [5]. However, the early onset of accelerated growth described in the chick model has not been replicated or studied in other species. Accordingly, the response of the vasculature at this early age is not predictable. To our knowledge the only study to explore loading conditions in the prenatal heart is our work on embryonic day 12 rat hearts which were grafted in oculo in adult rat hosts [7]. Data from that study showed that 3 weeks after grafting this unloaded, beating heart had vascular densities which were similar to a full term (21 days) heart developing in utero. Thus, the slow myocyte growth in this model was accompanied by a slow but proportional growth of the vasculature. However, this approach is limited, since the ventricles are not operating in a normal hemodynamic state.

The central question addressed in this study was: does an accelerated growth and enhanced ventricular mass due to an increased afterload in the embryo induce a comparable accelerated coronary vascular response? The second question concerned the morphogenetic changes in the ventricles. The ventricular walls are initially heavily trabeculated with the intervening spaces constituting a sinusoidal system which provides oxygen and nutrients to the myocytes prior to the establishment of coronary arteries [7–10]. Thus, we questioned whether pressure overload and the consequential accelerated growth influences the modeling of the trabecular and compact regions of the ventricles.

2. Methods

2.1. Experimental protocol

All protocols used in this study conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The model of pressure overload used in this study has been previously described [6,10] and is summarized as follows. Fertilized White Leghorn chicken eggs were incubated, blunt end up, to HH stage 21 (3.5 days) of a 21-day incubation period. We reached the embryo by making a hole in the shell, and incising the inner shell membrane. The outflow tract of the heart was constricted by tying a loop of 10-0 nylon suture at the midpoint of the conotruncus. At stage 21 the outflow tract and vessels are semitranslucent and thus we were able to distinguish the conotruncal valves, which mark the root of the conotruncus. The outflow tract ends at the base of the aortic arches, which emerge at the neck region. The procedure was performed under a photomicroscope attached to a videocassette recorder. Sequential video fields of the loop diameter and the diameter of the conotruncus at the suture region were planimetered using a video frame grabbing board and video software. Accordingly, the loop diameter was adjusted to the diameter of the outflow tract. In sham-operated embryos the suture was placed in position but not tied. Normal embryos were unoperated. The window in the shell was sealed with paraffin film, and the egg was returned to the incubator. Hearts from embryos of various incubation periods were perfus-®xed in diastole, via a microcannula placed into the ventricle. The diastolic state was maintained by perfusing 0.025 μg verapamil in a 2.5% glutaraldehyde solution buffered with 0.1 M sodium cacodylate. The heart was then excised, and the ventricles were weighed, post®xed in 1% osmium tetroxide and embedded in Spurr’s plastic.

2.2. Light microscopy and morphometric analysis

Sections, 1 μm in thickness, were cut on a ultramicrotome and stained with Richardson’s solution. Sections from several levels of the ventricles were used for a subjective analysis of morphological characteristics, while sections from the midpoint between the base and apex were used to assess the thickness of the compact layer of the myocardium. This was accomplished by projecting images of the left ventricular free wall onto drawing paper with a microprojector and tracing the outlines of the ventricular wall. Subsequently, equidistant points were placed along the epicardial–myocardial border and distances from these points and the nearest points of the endocardial endothelium were measured. The mean distance for each specimen was based on an average of 33 measurements representing over 1 mm of the epicardial–myocardial border.

Serial sections of normal hearts were prepared from JB-4 blocks on a Reichert microtome and stained with eosin and hematoxylin and representative sections were photographed. Particular attention was paid to the basal half of the heart and the formation of the coronary arteries and the time of coalescence with the aorta. Coronary vessel diameters in the stage 36 hearts were obtained from tracings (×552) of microprojected images.

2.3. Electron microscopy and morphometric analysis

Ultrathin sections were prepared with a diamond knife on a Reichert Ultratome, placed on either single oval slot or 100 mesh cooper grids coated with a formvar film. The sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-7000 electron microscope.
For quantification of vascular density, specimens were photographed at ×1000 and micrographs prepared at a total magnification of ×2500. At least eight fields (of the compact region), representing over 10 000 µm² of tissue, were photographed from two or more blocks. Volume density of vessels <12 µm was determined, as previously described [4], by point counting of vessels using an overlay grid with intercepts every 6 mm (1008 points/micrograph). Nonvascular cells were point-counted using an overlay grid with intercepts every 12 mm (252/micrograph) and the counts multiplied by 4 in order to express vascularity as percent of tissue (excluding extracellular space). Vascular volume density (V̂v) is derived from the relationship: 
\[ V̂v = \frac{V_{v,0}}{V_t} = \frac{V_{v,1}}{P_{v,1} \cdot P_t}, \]
where \( V_{v,0} \) is the number of points falling on vessels and \( P_t \) is the total number of points falling on various cells plus vessels. The basal half of one group of overloaded and sham HH 36 hearts was dehydrated, sputter-coated with gold and studied with a scanning electron microscope (Hitachi S-4000). The hearts were mounted so that they could be viewed from their cut surface. Micrographs from these hearts were used to assess intergroup differences in the spongy and compact regions of the ventricles and to measure the thickness of the compact region.

### 2.4. Statistical analysis

The data were analyzed by analysis of variance and a Bonferroni adjustment for multiple comparisons. A p-value <0.05 was selected to test for significant differences between groups.

### 3. Results

#### 3.1. General effects of conotruncal banding

Data in this study are based on 42 embryos. The survival rate of the chicks banded at stage 21 was 60% at stage 29 and 35% at stage 36. As in previous studies [6,10], we did not note any general abnormalities in the embryos which were banded. We verified the position of the ligature in each embryo studied. All of the loops were positioned well above the conotruncal valves.

#### 3.2. Ventricular morphogenesis

As previously shown by scanning electron microscopy, the smooth walled chick ventricle at stage 16 develops myocardial trabeculae by stage 18 which grow to form a mesh by stage 21 [11]. Thus, the ventricular wall at this point consists of an outer compact layer and an inner spongy layer. The spaces between the trabeculae are termed “sinusoids” and serve to minimize diffusion distances between blood in the ventricle and myocardial cells. The formation of the coronary vasculature is initiated when angioblasts form tubes by joining together in either (1) blood islands, or outside blood islands, or (2) by forming large vacuoles and thereby “hollowing out” to form a channel. These microvascular channels coalesce and branch throughout the compact region. Attachment to the aorta occurs via ingrowth of coalescing capillary plexus at about stage 32 or 33, as indicated by analysis of our serial sections and as previously demonstrated by others [12]. Formation of a media in the arterial system begins at this time, and follows a proximal to distal sequence from the root of the aorta. This pattern was confirmed in this study and is similar to our previous observations in rats [8]. At stage 36 the arterial tree is limited to the basal \( \frac{1}{4} \) of the heart. Such channels were not encountered in sections used for quantitative analysis. At the time of banding (stage 21) the avascular left ventricular compact myocardium is about two cell layers thick in the compact zone, has numerous long trabeculae, and the epicardium is thinner than that observed at successive stages (Fig. 1A). By stage 24 the compact myocardium is about 3–4 cells in thickness and the trabecular (spongy) layer has also expanded. Large vascular plexuses overlay the AV groove. By stage 29 the myocardium is about 5–7 layers thick, the trabeculae are relatively shorter and the epicardium is considerably thicker (Fig. 1B). The myocardium grows rapidly between stages 29 and 36 as the compact myocardium increases to 12–20 cells in thickness and the trabecule thick and shorten (Fig. 2A). By stage 36 the spongy region of the ventricle is markedly reduced. Capillaries are seen only within the compact region and the thicker trabeculae. The coronary microvasculature is connected to the aorta by stage 33, as previously noted [12], and as confirmed by us in this study by analysis of serial sections.

#### 3.3. Ventricular mass and morphology in overloaded heart

In the banded hearts, at stage 29, the increase in thickness of the compact region (Fig. 1C) was due to an increase in the number of cell layers in the compact region, i.e., about 7–8 cells were not uncommon. Thus, at least part of the increase in cell number, previously documented in this model [6], occurred transmurally in the left ventricle and contributed to the substantial (64%) increase in ventricular mass (Table 1).

In contrast, the pressure-overloaded hearts harvested at stage 36 had a smaller mass than the shams (Table 1). The compact region of the heart did not develop further, thus a major portion of the left ventricular wall remained as a spongy layer, as seen in both histological sections (Fig. 2B) and in scanning electron micrographs (Fig. 3). The thickness of the compact region was quantified by measuring the shortest distance from the epicardium (the border of the epicardium and myocardium) to the endocardium (Fig. 4A). At stage 29 the mean thickness of the compact
percentage of the myocardium remaining as the spongy (trabecular layer). In sham hearts at this stage only 24% of the left ventricular wall consists of spongy myocardium, while in overloaded hearts 33% remains spongy ($p = 0.002$). Moreover, the ventricles in the stage 36 overloaded hearts were more ellipsoid that the shams, and mean overall ventricular thickness, measured in scanning electron microscopic micrographs, was 24% less in the overloaded group at HH stage 36 (mean ± SEM: 116 ± 10 vs. 153 ± 8 μm). Accordingly, the persistence of the pressure overload was associated with reduction of ventricular growth between stages 29 and 36.

3.4. Vascular growth

Fig. 5 illustrates a capillary as seen with the electron microscope, and represents a portion of a field used to quantify vascularity. We selected fields from the compact region which bordered the epicardium. In order to minimize the inclusion of sinusoids in our measurements we excluded any region with an endothelial-lined channel with a diameter above 12 μm. This criterion was based on preliminary experiments in which we traced structures which appeared to be vascular channels of this size or larger in serial sections and determined that they were continuous with the ventricular lumen. Our data on vessel volume percent (Fig. 6) and vessel numerical density (Fig. 7) indicate that vascularity was proportional to ventricular mass, i.e., vascular growth in the overloaded hearts was accelerated between stages 21 and 29 in order to compensate for the greater increase in ventricular mass in this group. Thus, vascular growth necessarily increased proportionally to the accelerated growth of the myocardium. The vasculature was qualitatively similar in overloaded and sham hearts, and evidence of cell damage or degeneration was lacking. Vascular volume percent in the overloaded and sham hearts in the stage 36 chicks was also similar (Fig. 6), but numerical density was higher in the overloaded compared to the sham hearts (Fig. 7). This finding indicates that the vessels in the overloaded hearts were smaller than in shams.

In order to assess the formation of the coronary arteries, we examined serial sections through the basal $\frac{1}{3}$ of the heart. The coronary arteries in sham hearts are continuous with the aorta at stage 32 or 33. In stage 36 sham hearts the coronary arteries extend down about $\frac{1}{3}$ of the distance between base and apex (Fig. 8A). They appear to branch only once or twice. The three banded hearts which were region is 27% greater in the overloaded than in the sham hearts. In contrast, by stage 36 the compact region mean thickness is 31% lower in the overloaded ventricles compared to the shams. Thus, the compact region of left ventricle expanded to a much greater extent in the sham than in the overloaded group. As illustrated in Fig. 4B, the lower growth rate of the compact region between stages 29–36 in the overloaded hearts resulted in a higher

Fig. 1. Light micrographs of the left ventricular wall of hearts from normal HH stage 21 (A), sham HH 29 (B), and overloaded HH 29 (C) chick hearts. In normal (nonoverloaded) hearts the thickness of the compact region (between arrowheads) increases approximately threefold between stages 21 and 29. In the overloaded hearts this increase is significantly greater (see Fig. 4) as a consequence of more cells. Bar = 100 μm.
Finally, we have shown that development of the coronary arteries is stunted in hearts which were banded.

4.1. Ventricular adaptations to embryonic pressure overload

Previous work by our group showed that between stages 21–29 ventricular pressure increased more than twofold, cardiac work increased about sevenfold, and left ventricular mass increased ninefold [13,14]. Subsequently, we found that when the outflow tract was banded at stage 21 ventricular dry weight increased about 40% above the sham-banded controls, and that this compensatory growth was due to myocyte hyperplasia since myocyte size and DNA:protein ratio were similar to the controls [6]. Moreover, overload did not affect embryo dry:wet weights, heart rate, cardiac output, or overall ventricular growth. Thus, accelerated ventricular growth during this period of time did not result in abnormalities in these structural and functional parameters.

In the current study we found that pressure overload during stages 21–29 increases the thickness of the compact region of the left ventricle as evidenced by an increase in epicardial to endocardial distance. In sharp contrast, persistence of the pressure overload to stage 36 alters left ventricular growth and morphology, i.e., the compact region grows more slowly and usually develops a more ellipsoidal shape than that of hearts with a normal afterload. This reduction in growth in the compact region resulted in a smaller area of compact myocardium and a greater area of spongy myocardium compared to the shams. Growth between stages 29 and 36 in the banded hearts was so markedly depressed that their ventricles were smaller than the shams. Thus, our experimental design has included two stages of pressure overload: (1) a compensated stage with acceleration of ventricular growth, and (2) a decompensated stage in which ventricular growth is decelerated compared to the nonoverloaded sham hearts.

The reason for the decelerated ventricular growth in
Fig. 3. Scanning electron micrographs of hearts from HH stage 36 hearts. Note that the compact region between arrowheads of the sham heart (A) is thicker than that of the pressure-overloaded heart (B). Sinusoids are indicated by arrows. The left ventricles of overloaded hearts are usually more ellipsoid than those of shams, as seen in these micrographs. At a higher power view of sham (C) and overloaded (D) hearts, the presence of sinusoids in the ventricular wall can be more clearly visualized (arrows). Note that sinusoids are closer to the epicardium in the stage 36 overloaded hearts (compared to shams), i.e., the compact layer is less developed while the spongy layer persists to a greater extent. In A, Bar = 1 mm; in B, Bar = 100 μm.

Fig. 4. (A) The mean thickness (±SEM) of the compact region of the left ventricular wall is illustrated for each of the four groups. Mean thickness was based on measurements of the shortest distance from the epicardial–myocardial border to the endocardium as derived from projected images of tissue sections. (B) Percent of the left ventricle composed of spongy myocardium. These values are based on image analysis of slides of cross-sectioned left ventricle. Five to seven hearts were included in each group.

banded hearts between stages 29 and 36 is not evident. One possible reason could be the poor development of the coronary vessels and as a consequence limited O₂ availability to the compact region. Thus, the compact myocardium failed to expand because coronary perfusion was inadequate or failed to develop. Another possible reason for the decelerated growth is that the pressure overload increased as the band became more restrictive as the outflow tract increased in diameter, thereby exhausting the adaptability of the myocytes. This explanation, however, is less likely since myocytes maintained normal ultrastructural features.

4.2. Accelerated vasculogenesis/angiogenesis with accelerated ventricular growth

The accelerated ventricular growth between stages 21 and 29 was substantial (64% increase in wet weight) and occurred in a relatively short time span (2.5 days). The finding that coronary vascular growth matched the increase in mass indicates that the latter, by some yet undefined
mechanism, dictates the degree of vessel formation. In postnatal hearts the vascular response to ventricular hypertrophy is usually limited and/or requires a long period of time, and is dependent upon the stimulus evoking the ventricular enlargement (reviewed in Refs. [1,2]). The current study is the first to address adaptive vascular growth in embryonic hearts.

Vasculogenesis occurs as the compact region of the ventricle thickens, and is not seen in trabeculae until much later and only when they reach a certain critical thickness. Thus, vascularization occurs as myocardial cells become remote from the endocardium and their initial source of oxygen. Accordingly, hypoxia is one possible trigger of vascularization in the embryonic heart. Consistent with this hypothesis is the work of Hoper and Jahn [15] indicating that oxygen levels influence the vascular density of the
yolk sac of chick embryos, i.e., hypoxia increases vascular density, while hyperoxia decreases vascular density. Vascular endothelial cell growth factor (VEGF) mRNA is upregulated by hypoxia [16,17] and accordingly could serve as a primary inducer of coronary vasculogenesis. This growth factor and its receptors are expressed in early embryos [18]. Flk-1 (a VEGF receptor) mRNA expression has been found in all endothelial cells at all stages of development [19]. Thus, one possible explanation of the accelerated coronary growth in the hearts of pressure-overloaded embryos is that a relative hypoxia enhances VEGF expression and accelerates coronary growth in the compact region which initially expands more rapidly then that of the shams. Indeed, unpublished data on embryonic rat hearts from our laboratory show that the highest expression of VEGF mRNA is initially near the epicardium and corresponds to the first sites of vascular tube formation. While VEGF is a key vasculogenic/angiogenic factor, other growth factors may also play a regulatory role in accelerated myocardial vascularization. For instance, basic fibroblast growth factor is upregulated in the early stage of embryonic myocardial vascularization [8]. We have shown that both of these growth factors play a role in coronary vessel formation in vitro in the rat [20].

An unexpected finding was the decreased myocardial and vascular growth in the overloaded hearts between stages 29 and 36, i.e. heart mass increased only about 2.5-times compared to 6.2-times in the sham. Thus, vascular volume growth paralleled the increase in ventricular mass. This is evidenced by the fact that vessel volume density of the overloaded hearts was virtually identical to the shams at both stages of development. Consistent with this finding are data on capillary growth in normally loaded prenatal sheep hearts [21], which indicate that during the last 4 weeks of prenatal development a consistent intercapillary distance is maintained as capillary proliferation compensates for the myocardial cell hyperplasia and hypertrophy. Thus, cardiac mass and myocardial vascularity are closely linked during the embryonic period. This relationship is also evident in the distribution of the two main coronary arteries in humans [22]. Regardless of left:right ventricular mass ratio, the right coronary artery perfuses approximately the same fraction of the ventricular mass. Thus, the perfusion territory of a given vessel adjusts to the cardiac mass.

Although vessel volume percent was similar for overloaded and sham chicks at each stage studied, vessel numerical density was higher in the overloaded group at stage 36 compared to the shams. Normal growth between stages 29 and 36 had no effect on numerical density, and overload between stages 21 and 29 also failed to influence this parameter. We interpret the increased vessel numerical density in the overloaded hearts at stage 36 as evidence that vascular proliferation was not blunted to the same degree as myocardial growth between stages 29 and 36, while vessel size was sufficiently blunted to maintain a normal volume percent. In contrast, the coronary arteries, which form at stages 32 and 33, were compromised in the banded hearts. Their failure to become confluent with the aorta, or their failure to develop may be the consequence of (1) the enhanced pressure within the aorta and ventricle or (2) interference with migrating cells along the outflow tract or signals originating above the site of ingrowth of the vascular plexuses which merge to form the coronary arteries. While neural crest cell ablation leads to variance in their sites of origin, they still form at the appropriate time [23]. Therefore, defects in neural crest migration do not appear to be the cause of coronary artery developmental failure or inadequacy. The tunica media of coronary arteries is not derived from the cardiac neural crest,
although neural crest cells appear around the origin of the coronary arteries, but not until embryonic day 14 or about stage 40 [24].

In conclusion, our data define an interrelationship between myocardial mass and vascular growth. Vascular volume growth (1) accelerates when myocardial growth is increased and (2) decelerates when the rate of myocardial growth decreases. These growth modifications seen in this model of prenatal pressure overload affect primarily the compact region of the myocardium where most of the vascularization occurs. If similar mechanisms operate in human development, changes in embryonic cardiovascular function may have long term effects on myocardial formation and vascularization. Our second major finding indicates that outflow tract banding compromises the development of the coronary arteries.

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References