Myocardial Volume and Organization Are Changed by Failure of Addition of Secondary Heart Field Myocardium to the Cardiac Outflow Tract

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Cardiac neural crest ablation results in primary myocardial dysfunction and failure of the secondary heart field to add the definitive myocardium to the cardiac outflow tract. The current study was undertaken to understand the changes in myocardial characteristics in the heart tube, including volume, proliferation, and cell size when the myocardium from the secondary heart field fails to be added to the primary heart tube. We used magnetic resonance and confocal microscopy to determine that the volume of myocardium in the looped heart was dramatically reduced and the compact layer of myocardium was thinner after neural crest ablation, especially in the outflow tract and ventricular regions. Proliferation measured by 5-bromo-2'-deoxyuridine incorporation was elevated at only one stage during looping, cell death was normal and myocardial cell size was increased. Taken together, these results indicate that there are fewer myocytes in the heart. By incubation day 8 when the heart would have normally completed septation, the anterior (ventral) wall of the right ventricle and right ventricular outflow tract was significantly thinner in the neural crest-ablated embryos than normal, but the thickness of the compact myocardium was normal in all other regions of the heart. The decreased volume and number of myocardial cells in the heart tube after neural crest ablation most likely reflects the amount of myocardium added by the secondary heart field. Developmental Dynamics 228:152–160, 2003. © 2003 Wiley-Liss, Inc.

Key words: myocardium; neural crest ablation; secondary heart field; heart; development; MRI; myocardial volume

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INTRODUCTION

Cardiac neural crest (CNC) ablation in chick embryos results in the loss of cardiac outflow tract septation and malalignment of the outflow tract (Kirby and Waldo, 1995). However, long before these manifestations of congenital heart defects can be seen, the absence of CNC cells in the pharynx of CNC-ablated chick embryos results in primary myocardial dysfunction (Farrell et al., 1999). This dysfunction is accompanied by failure of the secondary heart field to add the definitive myocardium to the cardiac outflow tract (Yelbuz et al., 2002). These results suggest that the elongation of the outflow limb of the cardiac tube by the secondary myocardium is a critical step in the normal process of looping insofar as it provides myocardium that allows the outflow tract to achieve a length consistent with its role in attaining
normal alignment for wedging of the aorta, which is critical to establish continuity of the aorta with the left ventricle, and alignment of the outflow septum with the ventricular and atrioventricular septa (Yelbuz et al., 2002). However, it is still unclear how much myocardium is added to the outflow tract by the secondary heart field.

There are two ways to determine the amount of myocardium added from the secondary heart field. One is to permanently mark the cells in the secondary heart field, allow them to incorporate into the outflow tract, and measure the number or volume of labeled myocardial cells. Because we do not as yet have a technique that could reliably mark the cells of the secondary heart field, this method is currently impractical. The second method is to inhibit addition of cells from the secondary heart field and measure the amount of myocardium in the heart tube in the absence of these cells. This method is less accurate but should provide an estimate of the amount of myocardium rather than measuring outside dimensions.

Magnetic resonance microscopy (MRM) allows assessment of volume without introduced artifacts that accompany reconstruction of histologic sections (Zhang et al., 2003). We used MRM to perform three-dimensional (3D) morphovolumetric analysis of chick hearts at HH stage 22 and compared myocardial volumes in normal hearts with hearts from chick embryos after CNC ablation. This was coupled with confocal microscopy to assess the organization and thickness of the myocardial wall. We determined that the volume of myocardium in the heart was dramatically reduced and that the compact layer was thinner. These changes were not confined to the outflow myocardium, indicating that myocardial changes occur throughout the heart tube. At the same time, myocardial proliferation and cell size are increased during the looping period while myocardial cell death is absent in both sham-operated and CNC-ablated embryos. On incubation day 8, when outflow septation was nearing completion, the myocardial cell number and wall thickness recovered normal dimensions with the exception that the anterior wall of the right ventricle and the proximal outflow tract were significantly thinner in the CNC-ablated embryos. These results suggest that the secondary heart field adds a significant volume of myocardium to the heart and that failure of this myocardium to be added results in significant disorganization of the compact myocardium.

RESULTS

Myocardial Volume Is Decreased After CNC Ablation

We used MRM to determine the volume of myocardium in the chick heart at stage 22 in both sham-operated and CNC-ablated embryos. Myocardial volume as described

<table>
<thead>
<tr>
<th>Age</th>
<th>Treatment group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Ventricle weight (mg)</th>
<th>Cells/ventricle ($\times 10^5$)</th>
<th>Cells/mg ventricle</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID5</td>
<td>Sham</td>
<td>5</td>
<td>0.323 (0.053)</td>
<td>2.20 (0.24)</td>
<td>5.2 (0.9)</td>
<td>2.4 (0.3)</td>
</tr>
<tr>
<td></td>
<td>CNC-ablated</td>
<td>5</td>
<td>0.242 (0.028)$^b$</td>
<td>1.57 (0.13)$^b$</td>
<td>3.8 (0.2)$^b$</td>
<td>2.5 (0.3)$^c$</td>
</tr>
<tr>
<td>ID9</td>
<td>Sham</td>
<td>5</td>
<td>1.89 (0.23)</td>
<td>13.6 (4.2)</td>
<td>28.6 (7.7)</td>
<td>2.1 (0.2)</td>
</tr>
<tr>
<td></td>
<td>CNC-ablated</td>
<td>5</td>
<td>1.78 (0.28)</td>
<td>14.7 (2.4)</td>
<td>34.0 (4.5)</td>
<td>2.3 (0.2)</td>
</tr>
</tbody>
</table>

aData presented as mean (SD). ID, incubation day; CNC, cardiac neural crest.

$^bP < 0.05$ comparing Sham and CNC-ablated groups.

$^cP = 0.075$. 

<p>| TABLE 1. Myocardial Volume (mm$^3$) in Sham-Operated and CNC-Ablated Embryos at Stage 22 Measured in MRM Images$^a$ |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>n</th>
<th>OFT</th>
<th>Ventricle</th>
<th>Atrium</th>
<th>n</th>
<th>OFT</th>
<th>Ventricle</th>
<th>Atrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>5</td>
<td>191.8</td>
<td>449.2</td>
<td>318.6</td>
<td>CNC-ablated</td>
<td>5</td>
<td>74.0</td>
</tr>
<tr>
<td>% change</td>
<td>↓ 61.4</td>
<td>↓ 67.2</td>
<td>↓ 49.1</td>
<td>P value</td>
<td>0.001051</td>
<td>0.008714</td>
<td>0.040847</td>
</tr>
</tbody>
</table>

$^a$CNC, cardiac neural crest; MRM, magnetic resonance microscopy; OFT, outflow tract.
here is the volume occupied by cells comprising the myocardial wall. We found excellent correlation between images obtained from MRM with those obtained by routine histology (Zhang et al., 2003). The 3D MRM reconstructions of hearts from sham-operated vs. CNC-ablated embryos were made, and these could be viewed as sections in any plane (Fig. 1). In the frontal plane, these images illustrate a significant degree of cranial displacement of trabeculated myocardium from the right ventricle into the outflow tract in CNC-ablated embryos as reported previously (Yelbuz et al., 2002). This finding suggests rearrangement of the myocardium in the primary heart tube to compensate for the failure myocardium to be added from the secondary heart field. Average myocardial volume in sham-operated and CNC-ablated hearts is shown in Table 1. The reduction in myocardial volume ranged from 50 to 60% with the greatest reduction in the outflow and ventricular myocardium.

**Fig. 1.** Magnetic resonance microscopy (MRM) images from stage 22 sham-operated vs. Cardiac neural crest (CNC) -ablated embryos in frontal section showing the cranial displacement of trabeculated myocardium (arrows) from the ventricle into the outflow tract (OFT) as previously reported in histologic sections by Yelbuz et al. (2002). The white line indicates the level of the inner curvature.

**Fig. 2.** Histogram showing the distribution of cell sizes in sham-operated vs. cardiac neural crest-ablated (Exp) myocardial cells at stage 22.
Myocardial Cell Size Is Increased After CNC Ablation

The average area occupied by sham-operated myocardial cells was 14,785 (dimensionless units, n = 663) compared with 29,711 (n = 636) in the CNC-ablated embryos (P = 1.0 × 10^{-10}). A histogram showing the distribution of cell sizes can be seen in Figure 2. Because of the reduced volume and increased size of myocardial cells at stage 22 (day 4) after CNC ablation, we surmise that there are fewer cells.

Myocardial Cell Number Is Reduced at Incubation Day 5 but Recovers by Day 9

Because the number of cells in the CNC-ablated heart is less than normal at day 4 of incubation, we examined two additional days, one before cardiac septation (day 5) and a second after cardiac septation is complete (day 9), to determine whether recovery of cell number occurs. By using a routine histologic technique for assessing cell number, the number of myocardial cells per ventricle was obtained at two later times, incubation day (ID) 5 and ID9 (Table 2). Myocardial cell number continued to be reduced at ID5 but recovered by ID9 (Table 2), indicating a restitution of normal cell number during the septation phase of development.

Composition and Thickness of the Myocardial Wall Is Altered

The organization of the myofibrils allowed identification of different layers across the myocardial wall. The number of layers changed over time and by region of the heart from two at stage 12 to four at stage 22 (summarized in Fig. 3). At stage 12, a surface layer of the myocardium comprised the external layer of the heart wall and this was underlain by an inner layer of myocardial cells with mostly disorganized myofibrils abutting the cardiac jelly. At stage 14, the myocytes adjacent to the cardiac jelly became organized into a third distinct layer containing well-aligned myofibrils in the ventricular (midloop) portion of the loop. By stage 16, both inflow and ventricular portions of the myocardium had three layers while the myocardium of the outflow portion of the loop continued to have only inner and outer layers. In the CNC-ablated embryos, no third layer appeared in the outflow tract of stage 16 hearts. At stage 18, the inflow and outflow myocardium of sham and CNC-ab-
Fig. 3. A: Confocal images of whole-mount embryonic hearts from sham-operated embryos stained with rhodamine phalloidin at stages (St) 12, 14, 16, 18, and 22 show sites where measurements of myocardial thickness were made. Rectangles demarcate the imaging sites for each region of the heart: o, outflow tract; m, midloop (ventricle or prospective ventricular area); i, inflow region. B–D: The average thickness of the myocardial wall, measured by focusing through the myocardial depth with a confocal microscope, was thinner in cardiac neural crest (CNC)-ablated embryos. B: The outflow tract myocardium of CNC-ablated embryos was significantly thinner at all stages. C,D: Although the myocardium was thinner in the ventricular and inflow regions, the reduction was not significant, except at the earliest times.
lateral hearts showed three layers, whereas the ventricular portion of the loop began to show a fourth, trabeculated layer. A definitive compact myocardium was present at stage 22 that was composed of the three outer layers. The fourth trabeculated layer had become much thicker than the compact layer.

When focusing through the thickness of the myocardial wall, there was a general impression in CNC-ablated embryos that the depths of the myocardial wall and its individual layers had decreased and that there were fewer myofibrils. To confirm that the myocardium was thinner in the CNC-ablated embryos, the width of the myocardial wall was measured in the inflow, midloop, and outflow portions of the heart loop by using confocal microscopy. In all stages examined, there was a reduction in the thickness of the myocardial wall in all regions of the CNC-ablated hearts (Fig. 3). This was most apparent in the outflow tract in which the average width of the myocardial wall decreased significantly over time (39%; Fig. 3B). The ventricular (midloop) and inflow regions of the heart loop suffered a much smaller decrease in myocardial wall thickness (17% and 15%, respectively; Fig. 3C,D).

Ventral Wall of the Right Ventricle and Outflow Is Thinner after CNC Ablation

To determine whether the reduction in the myocardial wall during looping impacted the postseptation heart, we compared the compact myocardial layer of incubation day 8 CNC-ablated and sham-operated hearts. Measurements were made of the ventral, dorsal, and lateral right ventricular walls, the left ventricular free wall, and the ventricular septum (Fig. 4). Most of the measurements from the myocardium of the CNC-ablated embryo were similar to those from the sham-operated embryo. However, the ventral wall of the right ventricle and the proximal outflow tract were significantly thinner in the CNC-ablated hearts (Table 3).

Myocardial Proliferation Is Altered After CNC Ablation

Because proliferation of cardiomyocytes in the primary heart tube would have a significant impact on our assessment of myocardium added from the secondary heart field, we examined 5-bromo-2'-deoxyuridine (BrdU) incorporation as a measure of cell division. Significantly, more BrdU-labeled cells were seen in the myocardium of the outer curvature than the inner curvature in all regions of the heart loop in both CNC-ablated and sham-operated embryos between stages 12 to 18. Within the outer curvature itself, differences were apparent in BrdU labeling in the CNC-ablated vs. sham-operated embryos. At stage 16, proliferation in the outer curvature of the CNC-ablated hearts was significantly higher than that in sham hearts (Table 4). No differences were seen in the BrdU incorporation in endocardium or epicardium between the CNC-ablated and sham-operated embryos. As noted previously (Waldo et al., 1999), the thickness of the cardiac jelly was variable in the experimental embryos, whereas it was uniform in sham-operated embryos.

While myocardial proliferation was increased in CNC-ablated hearts at stage 16, cell death was normal at all stages, i.e., in both the sham and CNC-ablated hearts, there was a complete absence of dying cells from the outflow and ventricular regions of the myocardium. In the inflow myocardium, a few dying cells could be seen in the atrioventricular canal and sinus venosus (data not shown). These results are consistent with those of Watanabe et al. (1998), who found that there was no cell death in the looping chick heart before stage 22.

DISCUSSION

To understand how the heart is affected when myocardium fails to be added from the secondary heart field, we have examined myocardial development from several perspectives by performing tests to determine the volume of myocardium, myocardial proliferation, cell death, and organization. By using 3D high-resolution MRM, we were able to determine that the myocardium derived from the primary heart fields and comprising the heart tube until looping, was significantly reduced in all portions of the tube but most severely in the ventricle and outflow regions where myocardium should be added from the secondary heart field. We have shown previously that the length of the tube is shorter and straighter in living embryos (Leatherbury et al., 1999) after CNC ablation but appears to have a normal length in postmortem specimens even though the configuration of the outflow tract is abnormal (Waldo et al., 1999; Yelbuz et al., 2002). This finding suggests a rearrangement of myocardial cells in the looping heart tube to compensate for the lack of myocardial cells that should have been added from the secondary heart field. This rearrangement is further substantiated by the presence of trabeculae at the base of the outflow tract seen in this and a previous study (Yelbuz et al., 2002).

Assessment of the myocardial wall confirmed that the compact myocardium is thinner in the CNC-ablated hearts during the looping period with the greatest effect in the ventricular and outflow myocardium. At the same time, myocardial cell size is larger, indicating that there are significantly fewer cells in the CNC-ablated hearts, as might be predicted by the failure of myocardial cells to be added from the secondary heart field. This finding is confirmed by actual myocardial cell counts at incubation day 5, showing that the myocardial cell population is still below normal. Thus, we believe that the myocardial cells in the primary cardiac tube, i.e., those derived from the primary heart fields, respond to the lack of addition of myocardium from the secondary heart field by rearranging a smaller number of larger cells in an attempt to compensate for the missing cells.

We have noted previously some other changes in the myocardium after CNC ablation. At the microscopic level, myofibrillar disorganization could be seen in the outflow myocardium at stages 14–18 (Waldo et al., 1999). Of interest, although a prolifera-
tive response might have been predicted as a compensatory response, proliferation was abnormally enhanced at only one stage during looping. The distribution of the cardiac jelly was irregular, causing changes in the shape of the heart loop (Waldo et al., 1999). Here, we show that myocardial proliferation is normal, except at stage 16 when it is up-regulated in the entire heart tube. Our data are consistent with that of Sissman (1966) and Stalsberg (1969), who showed that the primary heart field and looping heart in avian embryos undergo waves of stage-correlated synchronous regional proliferation. Although up-regulation of myocardial proliferation after CNC ablation for a few hours might serve to restore some of the myocardial cell numbers, it obviously is not enough to reconstitute a normal population of myocardial cells. This up-regulation results in a dramatic decrease in myocardial volume at stage 22. Because myocardial cell death is absent, the missing volume of myocardium is due to the failure of myocardial cells to be added from the secondary heart field.

Even though the number of myocardial cells is still below normal at ID5, by ID9 the number of myocardial cells is normal. Because the ventricular wall thickness at ID8 was normal except for the anterior wall of the right ventricle and the cell number is normal at ID9, the inference can be made that the cell size is restored to normal. How does the normal myocardial cell number relate to the thin anterior right ventricular
wall at day 8? It is most likely due to the finding that the anterior wall of the right ventricle is a small proportion of the entire myocardium and the margin of error in estimating the total number of myocytes in the ventricles is too large to distinguish this regionally localized thinning of the myocardial wall.

A remaining question to be answered is why the myocardium of the secondary heart field is not added to the heart after CNC ablation because the CNC would not be in proximity to the secondary heart field in an intact embryo. Although more experiments will be needed to determine the answer, we have shown previously that CNC alters the availability of FGF8 in the pharynx (Farrell et al., 2001). Presumably, the commitment and/or differentiation of myocardial cells from the secondary heart field is influenced by a variety of signals that emanate from the pharynx (Waldo et al., 2001). CNC could alter the availability of such signals, which would be in superabundance in its absence. The identity of these hypothetical factors is still to be determined.

**EXPERIMENTAL PROCEDURES**

**Embryo Preparation**

CNC-ablated and sham-operated embryos were prepared as described previously (Waldo et al., 1999). Embryos were collected at stages 12, 14, 16, 18, 22, and ID8. Embryos used for myocardial proliferation studies were treated in ovo 1 hr before collection with 20 µl of a 40 mM BrdU solution according to the method used by Brand-Saberi et al. (1995). Embryos collected for confocal microscopy were placed in 1.8% phosphate buffered saline (PBS)-buffered potassium chloride solution (243 mM) until the hearts ceased beating in diastole. The embryos were staged by counting somites or examining external morphology after the extraembryonic membranes were removed (Hamburger and Hamilton, 1951). They were then rinsed in saline and fixed overnight in 2% paraformaldehyde in 0.1 M Hepes buffer, ID8 embryos were fixed overnight in 4% paraformaldehyde in PBS.

**MRM**

Sham-operated and CNC-ablated embryos were harvested at stage 22–23 (day 4). The fixative preparation, perfusion, and fixation of embryos as well as the technique for MRM and imaging volume rendering and histology have been described in detail (Zhang et al., 2003).

**Myocardial Proliferation**

Five CNC-ablated and sham-operated embryos were examined at each of the stages. The number of BrdU-positive cells per 50 myocardial cells was counted from digital photomicrographs of transverse sections stained with anti/BrdU and hematoxylin.

**Cardiac Morphometry**

Confocal microscopy (Bio-Rad Laboratories, Inc., Hercules, CA) was used to measure variations in the thickness of the myocardium. The myocardial myofibrillar organization was visualized by using rhodamine phalloidin. Measurements of the myocardial thickness were made on five CNC-ablated and sham-operated embryos from each stage. Stacked images consisted of 2–10 scans, each made with a Z-step of 0.25 microns. The myocardial thickness was determined by multiplying the number of Z-steps needed to focus through the myocardium by the depth of the Z-step.

Myocardial volume was measured in MRM images by using ImageJ software available from NIH. The myocardium was outlined manually by two independent observers to obtain the area in each visual section.

To compare the myocardial thickness of five ID8 sham-operated and CNC-ablated embryos, transverse sections at the ventricular level (7 µm thick) were stained with myosin heavy chain antibody MF20 (Developmental Studies Hybridoma Bank, Iowa City, IA). SPOT software was used to measure and document the thickness of the myocardium.

**Immunohistochemistry**

BrdU was visualized by using the ZYMED Staining Kit (ZYMED Laboratories, Inc., San Francisco, CA). Cell death was assessed by using the APOPTAG Apoptosis Detection Kit (Oncor, Intergen). The monoclonal antibody for myosin heavy chain MF20 was applied to ensure that only myocardial cells were assessed (Yelbuz et al., 2002). For confocal microscopy, fixed whole-mount embryos were stained with rhodamine phalloidin as described previously (Price et al., 1996).

**Myocardial Cell Size**

Myocardial cell size was measured at stage 22 by using myocardial cells that had been freshly dissociated using trypsin and DNase as reported in detail previously by Creazzo (1990). At stage 22, the heart contains few other cell types and these can be recognized in dissociated cell preparations as spherical, nonpigmented, cells with obvious inclusions. Cell size was measured on 636 cells from three CNC-ablated hearts and 667 cells from four sham-operated hearts by using ImageJ software.

Number of myocardial cells per ventricle was obtained at incubation days 5 and 9 by using the method of Anversa and colleagues (1990) as previously described (Creazzo et al., 1994) and summarized here. The hearts were trimmed of the atria and great vessels, and the ventricles weighed and fixed in 0.1 M Sorenson’s phosphate buffer (pH 7.2) containing 2% glutaraldehyde and 2% formaldehyde and embedded in Araldite. These blocks were sectioned at either 0.75 µm for cross-sections or 2 µm for longitudinal sections, and the sections were stained with toluidine blue. The number of myocytes per ventricle and the number of myocytes per unit volume of myocardium were determined by counting nuclei and measuring nuclear length in tissue sections (Anversa et al., 1990). Seventy-five files were measured from the ventricles of each heart. Because no difference was found in comparing apical, middle, and basal regions of the ventricles, the measurements reported here were made from approximately the middle region. The 0.75-µm sections
were visualized at $\times 1,000$ and the number of cross-sectional myocyte nuclear profiles ($N$) were counted in a defined area (A), overlaid by an ocular grid covering an area of $5,900 \, \mu m^2$. The values for $N$ and $A$ were averaged to determine the density of nuclear profiles per unit area of myocytes, $N_{(A)}$. The average nuclear length ($D$) was determined from 85 to 100 measurements of each tissue sample in the 2-$\mu m$-thick sections. The number of myocyte nuclei per unit volume of ventricle, $N_{(V)}$, was determined by using the equation $N_{(V)} = N_{(A)}/D$. Assuming a tissue density of 1.05, the volumes of the ventricles were calculated from their measured wet weights. The number of myocytes per ventricle was calculated from $N_{(V)}$.

Statistical Analysis

Two-tailed, unpaired $t$-tests were used to compare values for sham-operated vs. experimental hearts.

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