Shortened Outflow Tract Leads to Altered Cardiac Looping After Neural Crest Ablation

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Background—Congenital conotruncal malformations frequently involve dextroposed aorta. The pathogenesis of dextroposed aorta is not known but is thought to be due to abnormal looping and/or wedging of the outflow tract during early heart development. We examined the stage of cardiac looping in an experimental model of dextroposed aorta to determine the embryogenesis of this conotruncal malformation.

Methods and Results—Hearts were examined from neural crest–ablated embryos by using videocinephotography, scanning electron microscopy, and histological sections. The inflow and outflow limbs of the looped cardiac tube were misaligned with respect to each other, the inner curvature was diminished, and the outflow limb was straighter and displaced cranially in a manner consistent with diminished length. The altered length could be explained by a significant reduction in the number of cells added to the myocardium of the distal outflow tract from the secondary heart field.

Conclusions—The data are consistent with research showing that normal looping and wedging are essential for normal alignment of the aorta with the left ventricle. These processes are abnormal in neural crest–ablated embryos because of a failure of the outflow tract to lengthen by the addition of myocardial cells from the secondary heart field. (Circulation. 2002;106:504-510.)

Key Words: morphogenesis ■ heart defects, congenital ■ tetralogy of Fallot ■ aorta

Congenital malformations of the heart are observed in at least 1% of newborn babies, 10% of stillbirths, and an estimated 20% of spontaneous abortions.1,2 Conotruncal heart defects, in which there are anomalies of the ventricular outflow tract, are associated with the highest mortality risk in utero.3,4 Mortality remains high even after postnatal surgical repair,4a,4b thus making it imperative that we begin to understand the genesis of these defects.

Even though the study of heart development has undergone a renaissance in recent years, as reflected in many excellent books and reviews,5–7 we still do not understand the relation of malalignment defects to the processes of looping, convergence, and wedging. Because the segments of the cardiac tube are in sequence and are not septated, looping creates an inflow (proximal) and an outflow (distal) limb. The inflow and outflow limbs are brought together cranio-caudally in a process called convergence. After the limbs have converged, the inflow portion of the tube nestles behind the ventrally placed outflow limb to begin a new period of outflow tract adjustment called “wedging.” Final positioning of the outflow tract by wedging occurs only during the later stages of outflow septation.8–10 The outflow septum is correctly aligned if the aortic side of the proximal outflow tract (conotruncus) nestles between the atrioventricular valves.8–10

Malalignment of the outflow tract is seen as a nonwedged or dextroposed aorta. In babies, the dextroposed aorta is usually classified as tetralogy of Fallot or double-outlet right ventricle (DORV), depending on the degree of dextroposition. This type of malalignment can be produced in developing chick embryos by a variety of previously published experimental manipulations, including cardiac neural crest ablation (see review).10 After cardiac neural crest ablation, ≈90% of embryos surviving to days 8 to 11 have heart defects. The defects consist of conotruncal malformations that can be categorized into 2 major classes: persistent truncus arteriosus, in which the outflow septum is mostly or totally absent, and dextroposed aorta, in which a robust septum divides the aorta and pulmonary trunk but in which these vessels are malaligned regarding the ventricular septum as seen in dextroposed aorta.10 The same type of malalignment associated with dextroposition is a characteristic feature of the persistent truncus arteriosus associated with neural crest ablation. In a study by Farrell et al,11 outflow tract alignment was rescued by replacing the cardiac neural crest, even though the cells were not competent to complete outflow septation.

We have found that cardiac looping is abnormal after neural crest ablation and that the characteristics of abnormal
looping could be explained by a decreased incorporation of myocardial cells from a newly discovered secondary heart field to lengthen the outflow tract.

**Methods**

**Embryo Preparation**

Fertilized Arbor Acre chicken eggs (Seaboard Hatchery, Athens, Ga, or Gold Kist Hatchery, Siler City, NC) were incubated at 37°C and 97% humidity in a forced-draft incubator. Neural crest-ablated, sham-operated, and control embryos were prepared as described previously. The number of embryos used in each analysis is shown in the Table. For shell-less culture, the eggshell contents were transferred to a hexagonal polystyrene weigh boat (Fisher Scientific) at stage 12. The weigh boat was placed in a Petri dish with 0.5-cm distilled water and incubated at 37°C.

For scanning electron microscopy, gross morphology, and histology, embryos were removed from the shell and placed in buffered potassium chloride (243 mmol/L) until the heart stopped beating in diastole. Each embryo was staged according to the Hamburger and Hamilton staging procedure.

**Time-Lapse Videophotography**

At stage 14, embryos in shell-less culture were filmed for 10 seconds at 30-minute intervals over a 10-hour period with a digital video-camera (Kodak 100 HRC digital imager with a high-speed frame-grabber board) mounted on a stereomicroscope (Olympus Corp of America). The imaging system was connected to a microcomputer with a high-resolution monitor. Lighting was provided by 2 fiber-optic light sources. An additional light source was used for temperature maintenance. The imager filmed at a frame rate of 250 frames/s, which resulted in 100 frames per cardiac cycle for a typical embryo. The images were used to construct a short movie (QuickTime, version 4.1.2) of heart development showing a heart cycle recorded every 30 minutes over 10 hours (2400 image frames covering 20 events).

**Scanning Electron Microscopy**

The embryos were fixed overnight in paraformaldehyde/glutaraldehyde in cacodylate buffer. They were washed, dehydrated, dried in a Tousimis Sandri-790 critical point drier, mounted on stubs, coated with a 15- to 20-nm gold layer (Hummer gold sputterer, Technics, Inc.), and examined by scanning electron microscopy (XL-30 FEG scanning electron microscope, Phillips Electronics).

**Gross Morphology**

The external features of the heart were examined in sham-operated and neural crest-ablated embryos fixed in 4% phosphate-buffered paraformaldehyde. The embryos were photographed from the left side and from the front.

**Histology and Immunohistochemistry**

The embryos were immersion-fixed at 4°C overnight in 4% paraformaldehyde. The following day, they were dehydrated, cleared, and embedded in paraffin. The paraffin blocks were sectioned at 7 μm. The slides were postfixed in Bouin’s solution and stained with Weigert’s hematoxylin and Gomori’s 1-step trichrome stain.

For human natural killer cells defined by expression of the Leu-7 (HNK-1) and myofilament-20 (MF-20) immunostaining, paraffin sections were processed as described previously.

**Terminology**

The topographic relations in the embryonic heart are defined as follows: The inflow limb is the portion of the heart that gives rise to the inflow and atrial segment, atrioventricular canal, and presumptive left ventricle. The outflow limb includes the presumptive right ventricle and the portion of the cardiac tube that connects it with the aortic sac. The proximal portion of the outflow limb (connected to the embryonic right ventricular segment) is also known as the conus, whereas the distal portion (connected to the aortic sac) is mostly referred to as the truncus. In the present study, the parts of the early heart refer only to those segments that are enveloped by myocardium; thus, the aortic sac is not included as part of the heart (Figure 1D).

**Results**

**Time-Lapse Videophotography Shows Abnormal Alignment of Inflow and Outflow Limbs of Cardiac Tube After Neural Crest Ablation**

In control or sham-operated embryos, the inflow and the outflow limbs of the cardiac tube were aligned side by side such that the inflow was not visible from the right side of the embryo, whereas in the neural crest-ablated embryo, the outflow limb was displaced ventrally and cranially, so that
both inflow and outflow limbs were visible from the right side. The outflow limb of the loop appeared to be shorter in the experimental embryo (Figure 1C and 1D). These results are consistent with previously published data at stage 18.17

External Features of Heart Show Abnormal Configuration and Length of Outflow Limb

The gross morphology of the heart tube was examined at stages 18 and 22 in sham-operated and neural crest–ablated embryos. The inflow and outflow limbs of the heart were aligned side by side in sham-operated embryos (Figure 1E and 1G). The presumptive ventricles appeared U-shaped in the frontal plane, with the outflow limb arising almost vertically from the presumptive right ventricle, followed by a curving bend toward the midline. The outer curvature was relatively long, and the space in the inner curvature between the inflow and outflow limbs of the cardiac tube was relatively wide (Figure 1G).

In neural crest–ablated embryos (Figure 1F, 1H, and 1I), the space between the inflow and outflow limbs was diminished or reduced (Figure 1H and 1I). The outflow limbs appeared to be overall shorter in length and straighter than normal. As a result, the outflow limbs were ventrally and cranially displaced (Figure 1F, 1H, and 1I).

Alterations in the configuration of the outflow limbs were more easily visualized in scanning electron micrographs of stage-15/16 and stage-22 chick embryos (Figure 2A through D and Figure 2E and 2F, respectively). The space between the inflow and outflow limbs (inner curvature) appeared reduced. The outflow limbs appeared straighter than normal, ventrally and cranially displaced, and slightly displaced leftward. These changes were much more obvious at stage 15/16 than at stage 22, where the inner curvature and outflow

Figure 1. A through D, Images from time-lapse movie of stage-14 chick embryo in shell-less culture showing morphological changes in heart over 10 hours in sham-operated (A and B) and neural crest–ablated (C and D) embryos. Heart is depicted in right lateral view at end diastole at stage 14 (A and C) and 10 hours later at stage 16/17 (B and D). In normal embryo, inflow and outflow limbs of cardiac tube were aligned side by side such that inflow was not visible from right side of embryo, whereas in neural crest–ablated embryo, outflow limb was displaced ventrally/cranially and turned rightward so that both inflow (dashed lines) and outflow (continuous lines) limbs were visible from right side. Outflow limb of loop appeared to be shorter and straighter in experimental embryo. E through I, Left lateral (E and F) and frontal (G, H, and I) views of hearts at stage 18 from sham-operated (E and G) and neural crest–ablated (F, H, and I) embryos. Ventricular segments (*) are at deepest convexity of loop, with outflow tract (+) arising almost vertically from it. Note wide outer curvature, curved shape of outflow limb (short arrows), and space in inner curvature (long arrow) between inflow and outflow portion of heart (G). In neural crest–ablated embryos, the majority of hearts showed diminished space between inflow and outflow limbs (H and I; straight line indicates only direction of outflow limb and not its length). a indicates atrium; AAA, aortic arch arteries; dAo, dorsal aorta; o, outflow tract; and v, ventricle. Bar=0.29 mm (A through F), and bar=0.33 mm (G through I).

Figure 2. Scanning electron micrographs from frontal (A, B, E, and F) and right lateral (C and D) views of heart of sham-operated (A, C, and E) and neural crest–ablated (B, D, and F) chick embryos at stages 15/16 (A through D) and 22 (E and F) showing alignment of inflow and outflow limbs of early heart tube. Note relatively narrow width of ventricle at stage 22; right ventricle is shifted ventrally and leftward in such a way that it is located almost longitudinally below outflow limb (F). a indicates atrium; o, outflow tract; and v, ventricle. Bar=0.20 mm.
The present study shows that abnormal cardiac looping after neural crest ablation results from shortening of the outflow limb. The shortened outflow limb results from failure of myocardial cells to be added from the secondary heart field. It is not clear from the present study why cells in the secondary heart field behave abnormally after neural crest ablation. Shortening of the outflow limb results in a decrease in the inner curvature of the looped heart and displacement of the presumptive right ventricle cranially, as evidenced by the cranial shift of trabeculated myocardium in the outflow limb. Because of this shift and the potential failure of outflow myocardium to be added to the distal outflow limb during looping, it is not clear whether a definitive conus or truncus is added. In the absence of the conus, the ventricular outlets would be unable to form normally.

Different concepts have been proposed to explain the mechanism by which the ventricular outlets become connected to the outflow tract. Van Mierop and Gessner\textsuperscript{21} and Bouman et al\textsuperscript{22} previously described a medial shift of the truncus arteriosus and a rightward shift of the atrioventricular canal. In their view, if the medial shift of the conotruncal part of the heart does not occur, this part of the heart retains its original connection with the primitive right ventricle only (dextroposed), and the sole outlet from the left ventricle is

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**Histological Features of the Heart**

In transverse sections through the normal heart at stage 16, the inflow and outflow limbs of the cardiac tube were aligned side by side in the transverse plane, whereas in neural crest–ablated embryos, the outflow limb was shifted ventrally (Figure 3A and 3B). Compared with normal embryos, the length of the inner as well as the outer curvature of the cardiac loop was diminished, and the distances between the inner and outer curvature and between the presumptive atrium and the outflow limb of the presumptive ventricle were shorter in the experimental group (Figure 3C and 3D). Also, the myocardium appeared to be thicker and the cardiac jelly appeared more unevenly distributed in neural crest–ablated embryos (Figure 3D).

At stage 22, the outflow limb in neural crest–ablated embryos showed an acute bend to the left in transverse sections and appeared to be straighter than normal, displaced anteriorly and cranially, and to the left in relation to the atrioventricular endocardial cushions. The free space at the inner curvature appeared significantly diminished. The trabeculated myocardium was displaced cranially in the experimental group compared with the normal group (Figure 3E and 3F).

**Myocardial Cells From Secondary Heart Field Fail To Be Added to Outflow Myocardium in Neural Crest–Ablated Embryos**

The features of the heart suggested that the outflow limb was shorter in neural crest–ablated embryos than in sham-operated embryos. Several groups have recently identified a secondary heart field that adds myocardial cells to the distal outflow limb.\textsuperscript{18–20} HNK-1 is expressed by the cells in the second heart field as they move into the outflow limb.\textsuperscript{18} Most of the myocardial cells in the outflow limb at stages 16 and 18 were HNK-1 positive in sham-operated embryos (Figure 4A through 4C and Figure 5A and 5B). In contrast, the HNK-1–positive cells in the outflow myocardium and secondary heart field were dramatically reduced in neural crest–ablated embryos, suggesting that myocardial cells derived from the secondary heart field were not incorporated into the outflow myocardium (Figure 4D through 4F and Figure 5C and 5D). Although the myocardium of the sham-operated embryo was continuous around the distal outflow limb, as shown by MF-20 staining, gaps in the myocardial sleeve were apparent in the neural crest–ablated embryo at stage 16 (Figure 4D through 4F). Also, at this stage, the myocardial sleeve of the sham-operated embryos appeared filamentous, whereas that of the neural crest–ablated embryos showed amorphous hot spots of MF-20 staining (Figure 4G and 4H).

**Discussion**

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**Figure 3.** Configuration of inflow and outflow limbs of cardiac loop and alignment of outflow limb of cardiac loop with presumptive right ventricle shown in transverse histological sections through heart of normal (A, C, and E) and neural crest–ablated (B, D, and F) chick embryos at stage 16 (A through D) and stage 22 (E and F). Dorsal, ventral, left, and right are as noted in panel F. In addition to changes noted previously, myocardium appears to be thicker and cardiac jelly (CJ) appears to be more unevenly distributed in neural crest–ablated embryos (D). Note also that trabeculated myocardium is displaced cranially (above dashed horizontal line) in neural crest–ablated embryos (D). a indicates presumptive atrium (inflow limb of cardiac tube); Ao, aorta; EC, atrioventricular endocardial cushions; IC, inner curvature of cardiac loop; m, myocardium; OC, outer curvature of cardiac loop; o, outflow limb of cardiac tube; Ph, pharynx; and v, presumptive ventricle. Bar=0.200 mm.
formed by the primary interventricular foramen. Because the conus is incorporated into the right ventricle, DORV or single ventricle with dextroposed aorta results after incorrect or incomplete formation of the conus septum. The shortened outflow limb, observed in the present study, would have a more difficult time making the required leftward shift. However, because the outflow limb was shifted leftward in neural crest–ablated embryos, we believe that the event leading to malalignment is more likely to be related to the altered configuration of the inner curvature rather than abnormal shifting of the outflow limb.

Lamers, Christoffels, and colleagues advocated a radical revision in our view of ventriculoarterial alignment by proposing that early chamber formation is restricted to the outer curvature of the looped tube. The outflow tract is expanded leftward, whereas the atrioventricular canal is expanded rightward, allowing the right dorsal portion of the atrioventricular canal to become the interventricular foramen. This configuration would allow blood from the left ventricle to bypass the right ventricle altogether and flow directly into the conus. The expansion of the outflow and atrioventricular canal is accompanied by remodeling of the inner curvature. Thus, the inner curvature would be critical in alignment of the ventricles with the outflow tract. In neural crest–ablated hearts, the inner curvature is one of the regions most affected by failure of the outflow limb to lengthen.

Gessner proposed that manipulations interfering with normal bulbar rotation, forcing the truncus arteriosus to

Figure 4. A through F, Staggered transverse sections through junction of caudal outflow tract with pharynx in stage-16 sham-operated (A through C) and neural crest–ablated (D through F) embryos double-stained for HNK-1 and MF-20. HNK-1 shows position of migrating neural crest cells in sham-operated embryo, and their absence can be noted in neural crest–ablated embryo. Fewer HNK-1–positive cells can be seen in secondary heart field in neural crest–ablated embryo. Although myocardium of sham-operated embryo (A through C) is continuous around distal outflow tract, as shown by MF-20 staining, gaps in myocardial sleeve are apparent in neural crest–ablated embryo (arrows in panels D through F). G and H, Sections through outflow myocardium of stage-16 sham-operated (G) and neural crest–ablated (H) embryos stained with MF-20. Myocardial sleeve of sham-operated embryo (G) appears as continuous layer of filamentous cells, whereas that of neural crest–ablated embryo (H) shows cells containing amorphous hot spots of MF-20. Yellow color in panels A through F indicates cells positive for both MF-20 and HNK-1. Bar=0.100 mm (A through F), and bar=0.020 mm (G and H).

Figure 5. Transverse sections through secondary heart field in stage-18 sham-operated (A and B) and neural crest–ablated (C and D) embryos double-stained for HNK-1 (green) and MF-20 (black). HNK-1 is expressed in neural crest cells and identifies cells in secondary heart field in process of migrating into outflow myocardium. Bar=0.100 mm.
remain in a more anterior and rightward location than normal, lead to dextroposition of the aorta. Yasui et al, using a retinoic acid model of DORV and tetralogy of Fallot, proposed that the malalignment was caused by a decrease in the counterclockwise rotation of the distal outflow tract. It is possible that the shortened outflow limb associated with neural crest ablation would interfere with the normal counterclockwise rotation of the aorta and pulmonary artery in the final steps of wedging. Thus, the length of the outflow limb at the beginning of outflow septation may determine the correct positioning of the aorta. The ventral and cranial displacement of the outflow limb could also account for the defects in development of the ventricular septum and inflow tract that have been described after cardiac neural crest ablation.9,10 Marking experiments will be required to determine directly whether the shortened outflow tract fails to undergo appropriate torsion.

Although the marking experiments suggest that the definitive conotruncus is not present in the primary straight heart tube but is added secondarily during looping,27–31 the secondary heart field that provides myocardium to the outflow tract during looping has only recently been identified in chick and mouse embryos.18–20 It is still unclear how much myocardium is added to the outflow tract and which segments of the primary heart tube are affected by “secondary” myocardium. Our 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 the length required for it to attain normal alignment.

We used HNK-1 and MF-20 in this and a previous study as markers of migration and myocardial differentiation, respectively, by cells in the secondary heart field.18 These cells express HNK-1 as they begin to translocate into the distal outflow myocardium. As they differentiate the myocardial phenotype, they become positive for MF-20, which has been shown previously to bind the light meromyosin fragment of myosin heavy chain in vertebrate striated muscle.32

HNK-1 is a glycoprotein that is expressed by migratory neural crest cells, by cardiac myocytes as they differentiate into conduction myocardium, and by neuronal processes.33,34 It has also been described in the endocardium.35 Its role in migration and axon growth is not known, although blocking antibodies to HNK-1 stop neural crest migration.36 We do not know whether blocking HNK-1 by antibody would alter the incorporation of cells from the secondary heart field into the outflow myocardium.

The results of the present study lead to the suggestion that the addition of myocardium to the outflow tract is necessary for normal looping and wedging to occur. These processes are critical in establishing continuity of the aorta with the left ventricle and alignment of the outflow septum with the ventricular and atriocentric septa.

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