Neural crest cells are known to contribute to the normal architecture of the heart and aortic arch arteries. Ablation of neural crest cells over somites 1 to 3 in the chick embryo prevents conotruncal septation and results in persistent truncus arteriosus. To determine whether a deficiency of cardiac neural crest cells produces hemodynamic changes prior to the development of identifiable structural defects in the heart, we measured dorsal aortic blood velocity and vitelline artery blood pressure in lesioned and control embryos at a period of cardiac morphogenesis prior to septal formation. The internal diameter of the dorsal aorta at the level of the sinus venosus and the internal diameter of the aortic arch arteries at their midpoints were measured in embryos at Stage 18 of development using a filar micrometer eyepiece and a dissecting microscope. Embryos with neural crest lesions had significantly greater dorsal aortic blood flow velocity than control embryos. In addition, embryos lacking cardiac neural crest had significantly lower systolic and diastolic blood pressures than control embryos. There was no difference in heart rate, dorsal aortic diameter or internal diameter of the aortic arch arteries between lesioned and control embryos. Scanning electron micrographs revealed no gross morphological differences in cardiac looping or conotruncal wall development between lesioned and control embryos; however, embryos with cardiac neural crest ablations developed markedly hypoplastic 4th pharyngeal arches. This data suggests that hemodynamic changes precede the onset of structural heart defects in embryos with cardiac neural crest ablation. (Circulation Research 1986;59:545-550)

The role of hemodynamic molding in cardiac morphogenesis has long been of interest since Bremer described the presence of two spiral streams of blood flow in the heart during development and postulated on their influence. The two streams arise from the right and left vitelline veins and pursue separate courses through the heart, even before the onset of cardiac septation. This suggests a possible role of hemodynamic forces in determining the location of the septa but has not been proven. Recently, Yoshida et al reexamined intracardiac flow patterns in developing chick embryos and observed flow patterns that do not support the traditional flow-molding theory. We do not examine blood flow patterns in the present study. However, the present study shows that depleting the heart and particular aortic arch arteries of cells derived from cardiac neural crest can result in hemodynamic alterations in the young chick embryo, even before the onset of structural defects.

Materials and Methods

Incubation and Microsurgery

Fertilized Arbor Acre chicken eggs were incubated in a humidified atmosphere for 26–30 hours at 37.5°C. Chick eggs were opened and prepared for microsurgery at Stage 8 or 9 of development. The chick neural fold over somites 1 to 3 was ablated with an electric microcautery needle. The neural fold consists of the presumptive dorsal part of the neural tube, the neural crest, and some adjacent surface ectoderm. The chick neural fold was ablated with no further manipu-
lation of the embryo. Aside from the induction of specific cardiac lesions, this procedure has been shown previously not to interfere with gross morphological development of the embryo." After surgery, the eggs were sealed, returned to the incubator, and allowed to develop for an additional 2 days to Stage 18 of development." Embryos that received sham operations were processed in parallel with the microsurgically manipulated embryos. For sham embryos, the egg was opened, the embryo stained with Neutral Red and the vitelline membrane torn at the level of the occipital somites. The eggs were sealed with a glass coverslip and returned to the incubator. In order to minimize variability, all microsurgery was performed by the same individual (M.L.K.) and all measurements were made by the same observer (D.E.S.).

Velocity and Pressure Measurements

Embryos were removed from the incubator at Stage 18 of development and positioned in an insulated heated sand bath (37.5°C) to maintain embryonic temperature on a dissecting microscope stage; each embryo was exposed by removing the glass coverslip window placed on the shell following microsurgery. Two different groups of embryos were used for the measurement of blood velocity (Table 1) and blood pressure (Table 2). The velocity of dorsal aortic flow was measured with a 20 MHz directional pulsed Doppler velocity meter at the level of the sinus venosus, as previously described by Clark and Hu." The internal diameter of the dorsal aorta was measured at the level of the sinus venosus with a filar micrometer eyepiece calibrated against a 10-μm scribed glass standard.

We recorded phasic and electronically integrated mean dorsal aortic blood velocity. The heart rate was determined by counting phasic pulsations between time lines. To calculate blood flow, we used the equation, Q = TVπd²/4, where V is mean dorsal aortic blood velocity and d is aortic diameter." Mean dorsal aortic blood flow divided by heart rate yielded dorsal aortic flow per cardiac cycle.

Systolic, diastolic, and electronically integrated mean blood pressure were measured with a servo-null pressure system manufactured by WP Instruments." For each pressure measurement we inserted a 4- to 6-μm diameter tip, drawn glass micropipette electrode in a first-order vitelline artery. Any embryos that bled on insertion of the electrode were discarded from the study. We assumed that dorsal aortic pressure and vitelline artery pressure were similar." For both blood velocity and blood pressure measurements, individual recordings were made for a 3- to 5-minute period, and then three 30-second intervals were selected for computation of the mean. Blood flow to the head and myocardium originate before the point of measurement; therefore calculation of mean dorsal aortic blood flow from measuring mean aortic velocity underestimates cardiac output. Student’s t test was used to determine significant differences (p<0.05) between control and experimental values.

Measurement of Aortic Arch Artery Diameter

A representative number of control and experimental embryos was injected via a vitelline vein with 1-μl Pelikan India ink. The ink was allowed to circulate for 3 minutes. The embryos were fixed in situ with Carnoy’s fixative and dissected from the egg. Following immersion fixation, the embryos were dehydrated in alcohol and cleared in methyl salicylate: benzyl benzoate (1:1). This procedure permits visualization of the endothelial lining of the aortic arch vessels (see Figure 1). We measured the internal diameter of the aortic arch arteries at their midpoint using a dissecting microscope and a filar micrometer eyepiece. In both experimental and sham operated embryos, the second, third, and fourth pairs of aortic arch arteries were present at Stage 18 of development."
submersion in 2% osmium tetroxide in SPB for 1 hour. Specimens were again rinsed in three 15-minute changes of SPB and dehydrated in graded alcohols for critical point drying. After critical point drying, embryos were oriented on an SEM stub and sputter-coated with gold. Specimens were observed and photographed using a JEOL scanning electron microscope.

**Results**

Embryos with neural crest lesions had significantly greater mean dorsal aortic blood velocity than control embryos (Table 1) although there was no difference in heart rate or dorsal aortic diameter. In spite of the increase in dorsal aortic blood velocity, the dorsal aortic blood flow approached significance but was not significantly increased ($p<0.05$, Student’s $t$ test, Table 1). Embryos with neural crest lesions had significantly lower systolic blood pressures than control embryos (Table 2). Their mean and diastolic blood pressures were also significantly lower than control embryos (Table 2).

No differences were observed in the diameters of the respective right and left second, third, and fourth aortic arch arteries of the lesioned and control embryos (Table 3). An index of the total cross-sectional diameter of the second, third, and fourth aortic arch arteries was obtained by adding the values of all three arches (Total diameter index, Table 3). There was no difference in total diameter index between control and lesioned embryos (Student’s $t$ test).

Figure 2A illustrates the configuration of the heart loop of a normal Stage 18 embryo; 2B shows the left pharyngeal arch region of the same embryo seen in A. Figures 2C and 2D are of a stage-matched experimental embryo. Although the heart loop is grossly relatively normal in Figure 2C, the conotruncus appears less curved than that of the control and the fourth pharyngeal arches are markedly hypoplastic in the same experimental embryo (left arch region seen in Figure 2D).

The heart loop and dissected conotruncus of a Stage 18 control embryo are shown in Figures 3A and 3B, respectively. Figure 3B illustrates the normal appearance of mesenchymal cells in the wall of the conotruncus at Stage 18. The heart and conotruncal wall in Figures 3C and 3D, respectively, are from an embryo that had neural crest cells ablated over somites 1–3; the mesenchymal cells in the conotruncal wall appear to

**Table 3. Aortic Arch Artery Internal Diameter (mm)**

<table>
<thead>
<tr>
<th></th>
<th>Arch 2</th>
<th></th>
<th>Arch 3</th>
<th></th>
<th>Arch 4</th>
<th></th>
<th>Total diameter index</th>
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<tr>
<td></td>
<td>R</td>
<td>L</td>
<td>R</td>
<td>L</td>
<td>R</td>
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<td></td>
</tr>
<tr>
<td>Control (n = 15)</td>
<td>0.06±0.01</td>
<td>0.06±0.01</td>
<td>0.11±0.01</td>
<td>0.10±0.01</td>
<td>0.06±0.01</td>
<td>0.05±0.01</td>
<td>0.44±0.02</td>
</tr>
<tr>
<td>S1–3 (n = 11)</td>
<td>0.07±0.01</td>
<td>0.08±0.01</td>
<td>0.11±0.01</td>
<td>0.12±0.01</td>
<td>0.05±0.01</td>
<td>0.05±0.01</td>
<td>0.48±0.04</td>
</tr>
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</table>

The internal diameter of each aortic arch artery was measured at the midpoint of the artery in Stage 18 embryos. S1–3 designates experimental embryos in which the neural crest was ablated over somites 1 to 3 at Stage 8–9 of development. All values are the mean ± SEM; the internal diameter is indicated in millimeters. The total diameter index is obtained by adding the mean values of both right and left arteries for all three arches. No difference in diameter was observed between control embryos and lesioned embryos for the total diameter index or for either side of any aortic arch artery (Student’s $t$ test). R, right; L, left.
FIGURE 2. Scanning electron micrographs of a ventral view of the hearts (A,C) and a lateral view of the left pharyngeal arch regions (B,D) of a Control (A,B) and an Experimental (C,D) embryo, respectively. Both embryos are at Stage 18 of development. A: Normal looping of the heart tube. B: Normal left pharyngeal arch region of the same embryo seen in A. C: Heart-loop of experimental embryo appears grossly to be relatively normal although the conotruncal region appears slightly less curved than that of the control in A; the atrium collapsed during tissue preparation and is not causally related to the microsurgery. D: Aortic arch region of the same experimental embryo as seen in C displaying a hypoplastic left (and right, not shown) 4th arch caused by the bilateral ablation of neural crest cells adjacent to somites 1-3. 1a = maxillary process of the first arch, 1b = mandibular process of first arch; 2,3,4 = second, third, and fourth pharyngeal arches, respectively. At = primitive atrium, V = primitive ventricle, CT = conotruncus. Bar = .091mm.

Discussion

The basic hypothesis of the interrelation of structure and function in the developing cardiovascular system is addressed in this study. We have shown with the present data that embryos deficient in neural crest cells originating adjacent to somites 1 to 3 have altered hemodynamics during the course of cardiac morphogenesis. This population of neural crest cells is known to migrate to the aortic arch arteries and to the aorticopulmonary septum. The neural crest cells over somites 1 and 2 migrate to the fourth pharyngeal arch and neural crest cells over somite 3 migrate to the sixth pharyngeal arch. Since the sixth pharyngeal arch was not yet present in the Stage 18 embryos used in this study, only the fourth pharyngeal arch of lesioned embryos should be deficient in neural crest cells. Although no difference in diameter of the fourth aortic arch arteries was observed between control and experimental embryos (Table 3), scanning electron micrographs indicate that the fourth pharyngeal arch is hypoplastic following ablation of the neural crest over somites 1-3 (Figure 2D).

Thompson and Fitzharris have reported that mesenchymal cells within the truncus are derived from two distinct sources: The first mesenchyme migrates caudally into the cardiac jelly of the distal truncus from the aortic arch region; a second mesenchymal population is derived from the endocardium. According to Van Mierop, at the embryonic stage used in this study the aorticopulmonary septum has not yet begun to develope.
op. The fact that the mesenchymal population in the conotruncus was normal at Stage 18 after neural crest ablation indicates that only a small population of neural crest-derived mesenchymal cells populate the conotruncal region. Their absence does not appear to affect the mesenchymal cells derived from endocardium. The present study suggests that the appearance of a hypoplastic pharyngeal arch (as seen in Figure 2D) is the first morphological marker to indicate the onset of development of a congenital cardiovascular defect caused by neural crest cell insufficiency. This implies that the cells which are lacking in the fourth pharyngeal arch (following neural crest ablation) are possibly from the same population as those described by Thompson and Fitzharris to first populate the wall of the truncus. Since cells are found in the wall of the conotruncus after neural crest ablation and coincident with a hypoplastic fourth pharyngeal arch (see Figures 3C and 3D), it appears likely that neural crest cells also migrate through the third pharyngeal arch en route to the conotruncus; preliminary studies have confirmed this to be true (Kirby, unpublished results).

It is noteworthy that at Stage 18 the fourth pharyngeal arch and respective arch artery are newly developed in the embryo and have not yet reached maximum size. Hence, hypoplasticity caused by a deficiency of neural crest cells in the fourth pharyngeal arch ought to be more apparent in an embryo at a later stage of development; this accounts for the observation that the fourth pharyngeal arch hypoplasia noted in this study is not more pronounced.

It seems remarkable that functional hemodynamic changes accompany the appearance of a hypoplastic pharyngeal arch at this early stage of development; this is particularly enigmatic since no differences in aortic arch artery diameter were observed between lesioned and control embryos. However, aortic arch artery diameters were obtained in fixed specimens and this is a possible misrepresentation of the status of arterial diameters in a living embryo. We suspect that aortic arch arteries lacking their normal complement of ectomesenchymal neural crest cells may have decreased resistance to blood flow induced by the lack of structural supporting cells (neural crest cells) in the aortic arch. Consequently, aortic arch artery diameters in the living embryo might be quite different from those measured in this study.

According to Poiseuille's law the resistance of a rigid tube equals $P/Q = (8 \times \text{viscosity} \times \text{length}) / (r^4 \times \eta)$, where $Q$ = flow, $r$ = radius, $P$ = pressure drop. In model conditions with laminar flow in a rigid tube, resistance is inversely proportional to the fourth power of the tube's radius. The most powerful determinant of resistance to flow is a change of the diameter of the arterial lumen. Of course, in the living embryo, arteries are not rigid and blood flow is unlikely to be laminar. However, Poiseuille's law can be used loosely to gain an understanding of functional hemodynamics. Therefore, it is valid to speculate that a decrease in frictional resistance to flow by the aortic arch arteries could occur if, indeed, arterial diameters in the living embryo are increased due to the deficiency of supporting cells following neural crest ablation. This could possibly account for the increase in dorsal aortic blood flow velocity in the lesioned embryos (Table 1). In spite of the increase in dorsal aortic blood velocity, the dorsal aortic blood flow is not significantly increased (Student's $t$ test). An alternative explanation for re-
duced resistance is that the ablation of neural crest cells somehow itself alters total systemic peripheral resistance; however no data is available to support this possibility.

The decreased systolic and diastolic blood pressures in the lesioned embryos (Table 1) are not easily explained. Our pressure values for control embryos, although somewhat lower, are comparable to those obtained by Clark and Hu11 in Stage 18 embryos. In addition, pulse pressures derived from both studies are similar for Stage 18 control embryos (Clark and Hu11, 0.30 mm Hg; present study, 0.27 mm Hg). In this study, lesioned embryos had a lower pulse pressure than control embryos (0.20 mm Hg vs. 0.27 mm Hg). Since measurements for control and experimental embryos were obtained in an identical manner with a comparable degree of difficulty, technical problems encountered during the pressure recording studies do not account for the lower systolic and diastolic pressures in lesioned embryos. The measurement of ventricular pressure in lesioned embryos might shed some understanding on the decreased pressure in the neural crest-ablated embryos.

This study indicates that functional changes in hemodynamics occur prior to any observable abnormal structural changes in the heart or aortic arch arteries of embryos with cardiac neural crest ablations. These embryos are known to eventually develop persistent truncus arteriosus;13,14 subsequently, this is the first evidence of altered hemodynamics induced in a reliable experimental embryo model eventually resulting in a specific form of congenital heart defect. We anticipate that the entire spectrum of congenital heart defects induced by ablation of neural crest cells will have related hemodynamic changes.

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References


Key Words • hemodynamics • chick • cardiac neural crest
Hemodynamic changes in chick embryos precede heart defects after cardiac neural crest ablation.

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