Cardiac Morphogenesis—Recent Research Advances

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ABSTRACT. It has been demonstrated recently that a specific region of neural crest contributes cells to the septa of the outflow tract of the heart. Removal of this region of cardiac neural crest prior to migration from the neural fold results in persistent truncus arteriosus in chick embryos. Removal of other regions of cranial neural crest results in double outlet right ventricle. Since double outlet right ventricle is produced by manipulation of noncardiac neural crest, this malformation is thought to be an indirect rather than direct effect of neural crest ablation. The cranial neural crest forms the walls of all of the aortic arch arteries and it is proposed herein that flow abnormalities are produced in the pharyngeal region by injury to the neural crest. These abnormal hemodynamic characteristics influence heart development. Cardiac neural crest seeds the heart with parasympathetic postganglionic neurons as well as ectomesenchyme. Removal of the cardiac neural crest results in cardiac malformations because of the decreased ectomesenchymal cells. However, the neural population undergoes regeneration and so the innervation of malformed hearts is morphologically normal. The mechanism for this regeneration is not understood. (*Pediatr Res* 21: 219–224, 1987)

Abbreviation

AV, atrioventricular

The pathogenesis of congenital heart defects has eluded clinicians and basic scientists for a long time. In the preface to his treatise "On Malformations of the Human Heart" published in 1858, Thomas Peacock says "There are few subjects which have attracted more attention in the profession than the irregularities in the development of the heart and large vessels" (1). More than a century has passed since Peacock's publication but many of his basic observations are still valuable.

Until fairly recently, the study of congenital heart defects was done inferentially by combining information gained at autopsy with what was known about embryogenesis of the heart. Shaner (2, 3) stands out in the literature on pathogenesis of congenital heart disease because he was able to find examples of several malformations in the early stages of development. In one study, he examined 20,000 pig embryos to find 48 abnormal hearts with some in the process of forming what would be transposition of the great arteries (3).

The development of experimental animal models of congenital

heart malformation has been very slow. The problems with these models have included the unpredictability of the result, low viability after the insult, and finally low incidence of malformation rate. Most investigators using these models have to maintain a precarious balance between mortality and incidence. This has made it very difficult to perturb the system and then follow the pathogenesis of a particular defect.

Several recent developments have dramatically expanded the range and utility of animal models which are now available for study of heart defects. These include the advent of genetic models (4, 5); new chemical teratogens which include bis-diamine (6), retinoic acid (7), and nimustine hydrochloride (8); and the discovery that neural crest ablation results in predictable heart malformations (reviewed below).

NEURAL CREST AND ITS DERIVATIVES

The neural crest exists only briefly in early embryonic development. It arises from the neural folds which develop from the lateralmost extent of the neural plate. As the neural plate closes to form the neural tube, neural crest cells are released from the neural fold (9). The factors wich control release and migration or translocation of the neural crest are not well understood. A prerequisite for release of neural crest cells from the neural fold seems to be the breakdown of the basement membrane underlying the neural crest cells. Following this event the crest cells extend processes and actively migrate away from the other components of the neural fold (9). It is not clear whether the cells actively migrate or are passively translocated to their final location, once they are free of the neural fold (10). Many extracellular components have been identified which are thought to be important in migration or translocation of the crest cells to their final destinations. Those molecules include fibronectin, hyaluronic acid, types I and IV collagen, and laminin (11). All are located in the neural crest migratory pathways.

The neural crest can be divided into two regions: cranial and trunk (11, 12). Trunk neural crest extends from the level of somite 5 to the caudal limit of the embryo (Fig. 1). It has received considerable attention from developmental neuroscientists because it provides all of the peripheral nervous system ganglia as well as Schwann and supporting cells for the entire peripheral nervous system, the adrenal medulla, and melanocytes (11–13). The cranial neural crest (Fig. 1) which extends from somite 5 cranially to the middiencephalon has the potential to form all of the same derivatives as trunk neural crest, but in addition it is capable of seeding mesenchymal cells to structures in the head and thorax (11, 12). These cells have been called ectomesenchymal because of their origin from the ectoderm. Cranial neural crest participates in development of the face and pharyngeal apparatus. Each pharyngeal arch (Fig. 2) and its accompanying pharyngeal pouch has unique derivatives including skeletal, muscular, and glandular tissues. Ectomesenchyme forms the skeletal derivatives and provides the connective tissue for the muscular and glandular derivatives (11, 12, 14). Each pharyngeal arch has
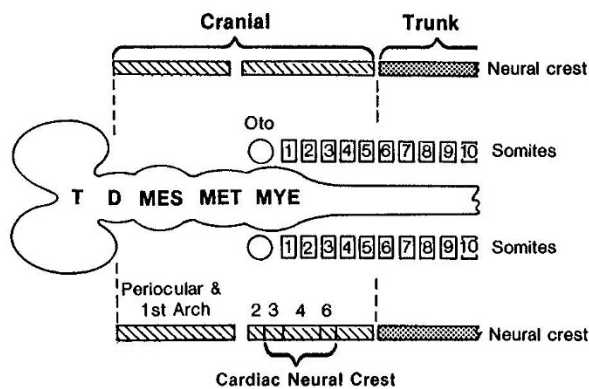
Fig. 1. Diagram illustrating the location of various parts of the neural crest and the areas of neural crest which seed the pharyngeal arches. Cranial neural crest extends from the mid diencephalon to the caudal part of somite 5. Trunk neural crest begins at somite 6 and extends to the caudal limit of the neural tube. The neural crest from the middiencephalon to the midmetencephalon seeds ectomesenchyme to the periocular and 1st arch regions. A small gap in the neural crest is present in the mid- to caudal metencephalon (14). The neural crest extending from the caudal metencephalon to midotic placode seeds arch 2. Arch 3 is seeded by crest extending from the midotic placode to the rostral limit of somite 1. The neural crest adjacent to somites 1 and 2 seeds arch 4 and the area adjacent to somite 3 seeds arch 6. The region which seeds arches 3–6 is referred to as cardiac neural crest because some of these cells migrate to the septa of the cardiac outflow tract. *T*, telencephalon; *D*, diencephalon; *MES*, mesencephalon; *MET*, metencephalon; *MYE*, myelencephalon; *OTO*, otic placode.
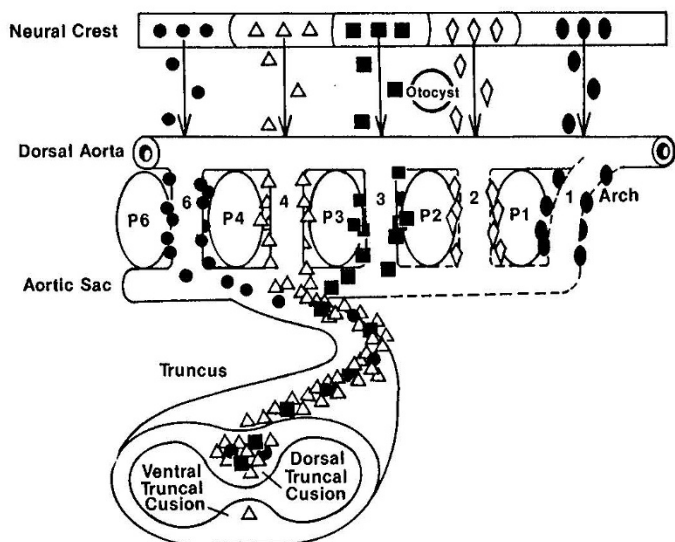

Fig. 2. Diagram illustrating the neural crest seeding pharyngeal arches 1–6. Ectomesenchyme provides the support for the aortic arch arteries which traverse the pharyngeal arches. Neural crest from arches 3–6 migrates into the outflow region of the heart. The ectomesenchyme in arches 1 and 2 does not go to the heart. Aortic arch arteries 1 and 2 disappear early in development (see Fig. 3) while arch arteries 3–6 persist (except for the left 4th arch) as permanent arteries of the thorax. Arch 4 provides the greatest quantity of cells to the outflow septa (see Fig. 4). In the chick heart, most of the neural crest cells are found in aorticopulmonary and truncal septa. The dorsal truncal cushion is the most active in septation and the greatest number of neural crest derived cells is found there. Pharyngeal pouches 1–6 are designed P1-P6.

its own aortic arch artery. These arteries appear in a specified sequence (Fig. 3) during development. Some are transient while others persist as definitive arteries in the adult. The tunica media of the aortic arch arteries which persist into adulthood consists of neural crest cell derivatives (15). The levels of cranial neural crest which seed ectomesenchyme to the various pharyngeal arches is shown in Figure 1 and the pattern of migration into the pharyngeal arches is shown in Figure 2.

In addition to sending ectomesenchyme to the pharyngeal arches, neural crest derived ectomesenchyme has been found in both the aorticopulmonary and truncal septa (16). Removal of cranial neural crest prior to migration results in a high incidence of congenital heart defects, some of which can be predicted by the location and extent of the lesion. These will be discussed in more detail below.

Although the ectomesenchymal component will be the major focus of the ensuing discussion of heart development, it is essential to remember that the heart is innervated by neurons derived from the neural crest. The parasympathetic postganglionic neurons arise from the same region of neural crest as the ectomesenchymal precursors destined for the outflow septa (17). Sympathetic innervation is provided by neurons arising from the trunk region of neural crest and has no associated ectomesenchyme (18). Sensory innervation of the heart is via the inferior ganglion of the vagus. Although the neurons of this ganglion arise from the nodose placode rather than neural crest, the neurons are supported by cells derived from the cranial neural crest (19). The relationship of the various components of cardiac innervation is shown in Figure 4.

Chimeric studies of neural crest derivatives. The study of a transient, migratory population of cells requires its own set of special techniques. Most of the recent work on neural crest has involved the use quail-chick chimeras. The interphase nuclei of cells of the Japanese quail *Coturnix coturnix japonica* contain a single central condensation of heterochromatin which is unique among avians (11). Quail cell populations can be transplanted to the early chick embryo and located at any time in the chick's later life.

Using this marking system, Le Lievre and Le Douarin (15) investigated the contribution of neural crest to the aortic arch artery derivatives. They found that the mesenchyme in the pharyngeal arches in a 4-day-old chimera was composed primarily of ectomesenchyme. The endothelium of the aortic arch arteries was composed of chick (nonneural crest) cells. In the later stages of incubation the walls of the common carotid arteries, brachiocephalic arteries, arch of the aorta, proximal pulmonary arteries, and ductus arteriosus were found to be ectomesenchyme which begins to differentiate into smooth muscle in the tunica media of these vessels on the 6th day of incubation. In addition to forming the walls of the aortic arch derivatives, ectomesenchyme has been found in the adjacent walls of the base of the aorta and pulmonary trunk (aorticopulmonary septum) and in the area between the semilunar valves and slighty inferior to the valves (truncal septum) (16). The conal septum does not seem to contain neural crest cells although the distinction between truncus and conus is difficult even with serial sections. Figure 3 shows the relationship of aortic arch artery development with some events including ventricular and truncal septal closure in heart development.

The level of origin of the neural crest cells which provides ectomesenchyme to the aorticopulmonary and truncal septa has been mapped (20). Premigratory neural crest corresponding to different pharyngeal arch levels in the quail were grafted homotopically into chick embryos. The neural crest which seeded arches 1 and 2 was not found near the heart. Neural crest seeding arches 3, 4, and 6 was found in the region of the outflow tract. Neural crest from arch 4 contributed four times as many cells to the outflow tract as arches 3 or 6 (Fig. 5).

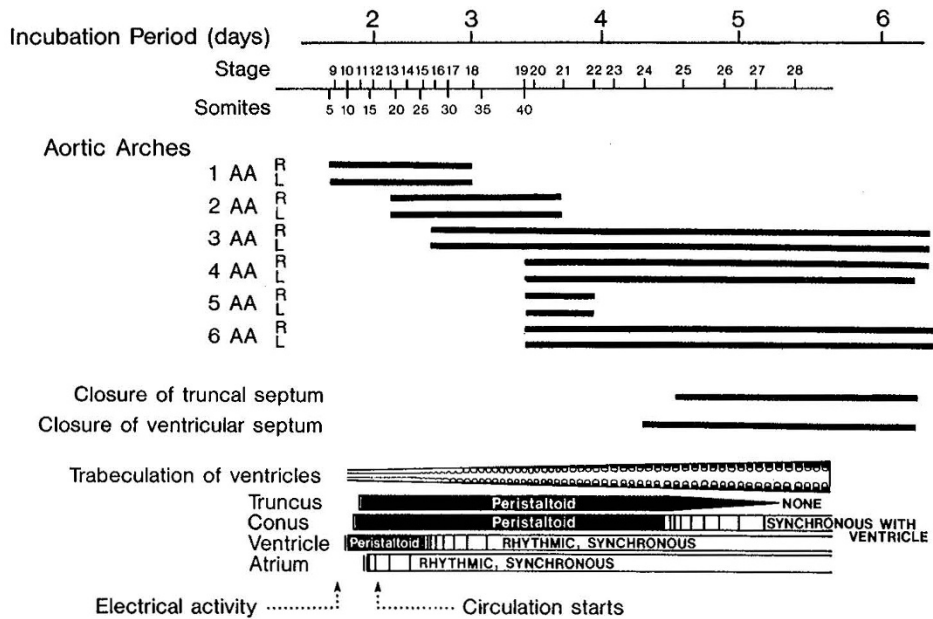Most of the neural crest cells in the truncus were found in

Fig. 3. A composite of information showing the relationship of various events in chick cardiovascular development. Development and disappearance of the aortic arch arteries (unpublished data) is shown in relation to functional events including outflow tract septation in the heart. Events in functional development of the heart are reproduced from Van Mierop (Ref. 36, reprinted with permission). Closure of the truncal septum is unpublished data. Closure of the ventricular septum is data from Roest-Wagenaar (37).
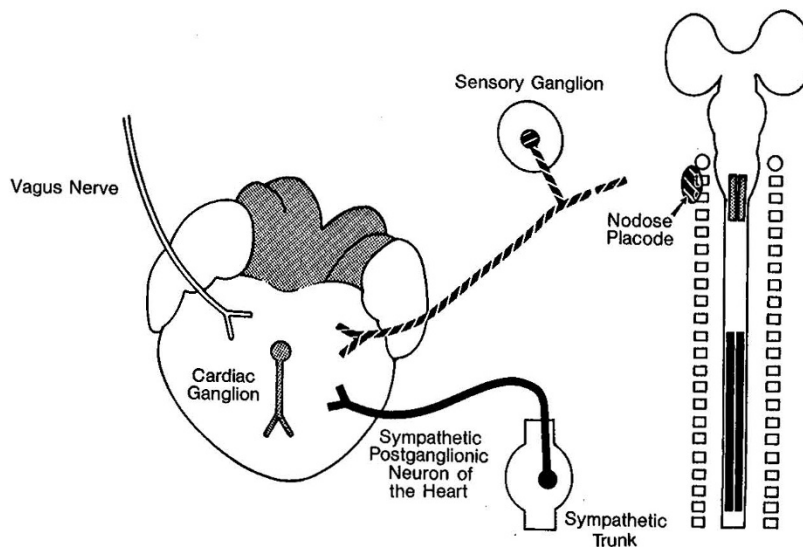

Fig. 4. Illustration of the relationship of cardiac innervation and its derivation. The parasympathetic (cardiac ganglion) postganglionic neurons are derived from neural crest adjacent to somites 1–3 (cardiac neural crest). The cardiac sympathetic postganglionic neurons (sympathetic trunk) arise from trunk neural crest adjacent to somites 10–20. Sensory innervation of the heart is thought to be from neurons in the inferior ganglion of the vagus which arises from the nodose placode. The neurons in this ganglion are supported by neural crest cells.

whorls of cells in the dorsal cushion. Few neural crest cells were found in the ventral cushion. Scattered clusters of cells could be easily recognized as cardiac ganglia derived from the same area of neural crest as the ectomesenchymal cells involved in septation (20). In the chick, the dorsal cushion is much more active in the process of truncal septation than the ventral cushion and so it is not surprising to find more ectomesenchymal cells dorsally (21). This is in contrast with the mammalian heart which has two large cushions which are equally active in septation. Since only neural crest destined for arches 3–6 can be found in the heart, this area has been called "cardiac" neural crest (Fig. 1) (20, 22).

NEURAL CREST ABLATION STUDIES

The role of neural crest in heart development was not entirely clear until ablation studies were performed. Ablation of premigratory cranial neural crest results in a variety of heart malformations. The type of heart malformation depends on the location and size of the neural crest ablation (22–24) but not on the age of the embryo when the ablation is performed. The age of the embryo when the ablation is performed influences the incidence of heart malformation without affecting the type of malformation (Fig. 6). The highest incidence occurs when the abla-
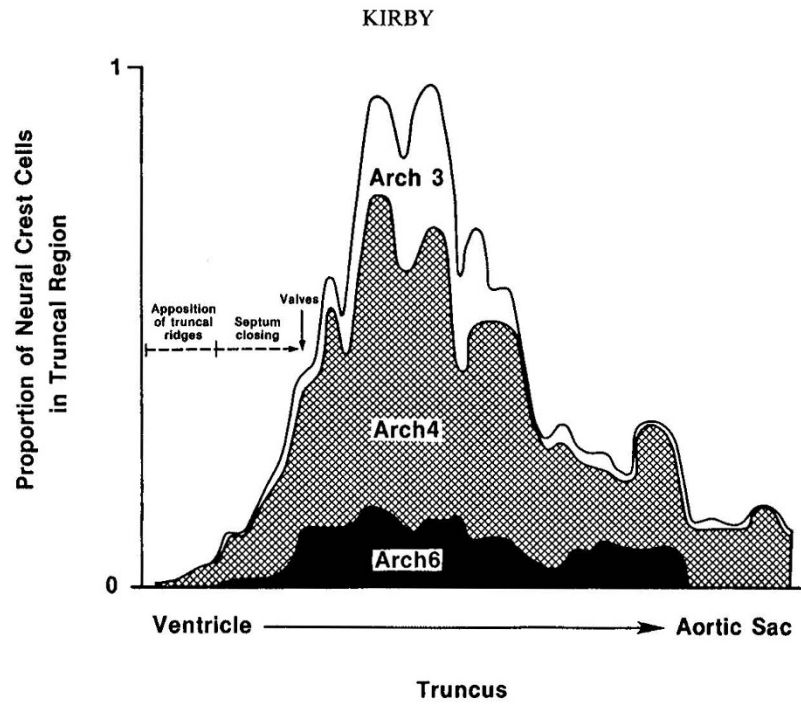
Truncus

Fig. 5. Diagram illustrating the distribution of ectomesenchyme derived from neural crest seeding arches 3, 4, and 6 to the truncal cushions. Downstream is indicated by the arrow on the abcissa. The major portion of cells (4:1 ratio) is derived from arch 4. Most of the cells are found in the dorsal truncal cushion before septal closure but some arch 4 cells can be found in the ventral truncal cushion (see Fig. 2).
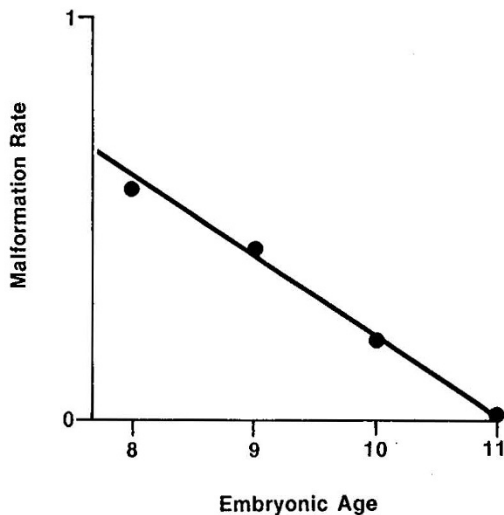

Fig. 6. Relationship of embryonic age to incidence of cardiac malformations produced by removal of premigratory neural crest. Stages (Hamburger and Hamilton) are shown on the *abcissa* which correspond to approximately 26–45 h (8–11, respectively) of chick development. Although increasing age influences the incidence of cardiac malformations, it does not appear to have much effect on the type of malformation produced.

tion is performed at stage 8 about 26–30 h of incubation (25) and no heart malformations are seen when the ablation is performed after stage 11 (about 40 h of incubation). This result is presumably due to the timing of neural crest migration. The neural crest cells in this region begin to migrate at about stage 9 (11).

Three heart malformations have been catalogued estensively after cranial neural crest ablation. These are ventricular septal defect, double outlet right ventricle, and persistent truncus arteriosus. Many other malformations are seen but have been less reliably correlated with the neural crest ablation. Other conotruncal anomalies seen have included transposition of the great vessels and defects similar to tetralogy of Fallot. Inflow tract anomalies which have been seen are: double inlet left ventricle, straddling right AV valve, right AV valve atresia, and persistent AV canal. The aortic arch artery derivatives show a variety of aberrant patterns after neural crest ablations (23, 24).

Persistent truncus arteriosus is seen only after ablation of all or portions of premigratory neural crest seeding arches 3, 4, and 6 (24). The ablation must extend at least 2 somite lengths (Fig. 7). This can be 2 unilateral somite lengths or 1 bilateral somite length (22). Extending the length of the neural crest ablation over arches 3, 4, and 6 increases the incidence of persistent truncus arteriosus. The area of neural crest ablation which produces persistent truncus arteriosus exactly corresponds with the area of neural crest which migrates to the aorticopulmonary and truncal septa in chimeric embryos (20). It is generally accepted that persistent truncus arteriosus results from failure of septation in the outflow area of the heart (26). Neural crest ablation studies suggest that the most likely pathogenesis of persistent truncus arteriosus in the chick embryo is a deficiency of neural crest-derived ectomesenchyme resulting in an undivided outflow tract (24).

An ablation of neural crest in the region of arches 3, 4, and 6 which is smaller than 2 somite lengths results in double outlet right ventricle (Fig. 7) (23). Double outlet right ventricle can also be produced by extensive ablations of neural crest over arches 1 and 2 (Fig. 7) (24). Persistent truncus arteriosus has never been seen after ablation of neural crest over arches 1 and 2 (24). Since double outlet right ventricle is produced by ablation of neural crest which does not seed the outflow septa (arches 1–2) as well as neural crest which seeds the heart (arches 3–6 or cardiac neural crest) this defect cannot be explained on the basis of decreased cells in the outflow septa. In double outlet right ventricle well-developed truncal and aorticopulmonary septa are present, but are misaligned with the ventricular septum. This defect can be produced by ablation of cardiac or noncardiac neural crest, and
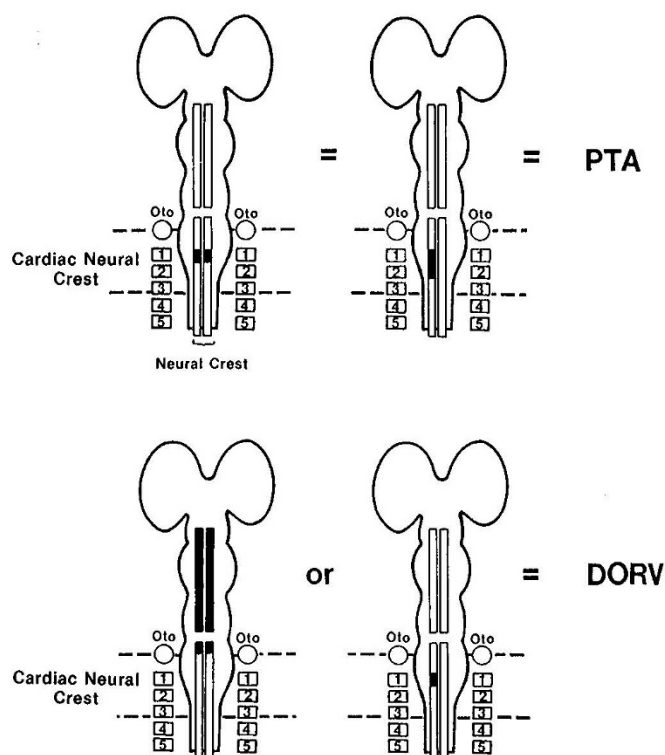
Fig. 7. An illustration showing the relationship of neural crest lesion size and placement in production of persistent truncus arteriosus (*PTA*) and double outlet right ventricle (*DORV*). A double somite length or greater of neural crest ablation in the cardiac region produces PTA. Lesions of neural crest cranial or caudal to the cardiac region never result in PTA. Large lesions of arch 1 and 2 neural crest or 1-somite length lesions in the cardiac area result in DORV.

thus the malformation must be an indirect effect of the neural crest ablation. The most likely place for an indirect effect to occur is in the aortic arch arteries. Indeed, aortic arch anomalies are common following cranial neural crest ablation (24). It is proposed that slight decreases in ectomesenchymal cells for arch arteries 3, 4, or 6 result in flow abnormalities which are transmitted upstream to the heart to cause misalignment of the outflow vessels and the ventricles. Decreases in ectomesenchymal cells around arches 1 and 2 cause similar hemodynamic changes and the result is the same.

In support of this hypothesis, Stewart *et al.* (27) have shown that hemodynamic parameters (blood pressure and velocity) are altered following neural crest ablation. The alterations can be detected several days before the outflow septa would normally appear. Since the ectomesenchyme surrounding the aortic arch arteries in the pharyngeal arches is derived from neural crest (Fig. 2), it may well be that flow is altered prior to any morphological changes in the heart.

Inflow tract anomalies, *i.e.* right AV valve atresia, straddling right AV valve, and double inlet left ventricle also occur frequently following ablation of cardiac or noncardiac cranial neural crest (24). Inflow tract anomalies induced by neural crest ablation are always associated with conotruncal anomalies. Thus it is probable that inflow tract anomalies are the result of indirect effects of the neural crest ablation rather than a decrease in the population of neural crest cells in the heart.

Any ablation of the cardiac neural crest will affect development of the parasympathetic innervation to the heart since the postganglionic neurons arise from this region (Fig. 3). Although it

was originally thought that the neural deficit was almost complete following cardiac neural crest ablation (17), later studies have shown a considerable regeneration of the neural component (28). Regeneration of the neural component occurs in the presence of severe cardiac malformations. This indicates that there is no regeneration of the ectomesenchymal component of the cardiac neural crest or that the timing of events in the lifespan of the ectomesenchymal cells is much more critical than that for the neural component. It is not known at present where the cardiac neurons arise when cardiac neural crest is removed.

It will be important to determine whether the regenerated cardiac neuronal population has the same characteristics as neurons derived from cardiac neural crest and whether normal control of cardiac function is established by the vagus in hearts with malformations induced by injury of cardiac neural crest. A complete understanding of the phenomena surrounding the cardiac innervation in neural crest-related heart malformations may be very important in the clinical management of children with certain types of heart malformations.

Syndromes of congenital malformations including heart. Chimeric studies have also shown that neural crest which seeds ectomesenchyme to the pharyngeal arches becomes connective tissue of the glandular derivatives of the adjacent pharyngeal pouches (29). The glandular derivatives of the pharyngeal apparatus include the thymus and parathyroids from pouches 3 and 4, connective tissue of the thyroid, and calcitonin-producing cells from the ultimobranchial body associated with the 6th pouch (11, 30). Removal of neural crest seeding arches 3, 4, or 6 causes hypoplasia or aplasia of the glandular derivatives of the associated pouches (31). Deficits of these glands accompany the cardiovascular malformations discussed above.

This finding is important because it offers an explanation for the pathogenesis of many syndromes which involve complexes of malformation involving pharyngeal arch derivatives and the cardiovascular system. The prototypic example in humans is DiGeorge syndrome. Van Mierop and Kutsche (32) have proposed that DiGeorge syndrome is due to abnormal developmental processes involving the neural crest because of the high incidence of persistent truncus arteriosus and interrupted aortic arch type B associated with DiGeorge syndrome.

Understanding the importance of neural crest to development of the heart, pharyngeal apparatus and face provides a useful background for understanding several teratogens which affect the cardiovascular system. Alcohol exposure in mice causes a complex of malformations involving the face, heart, and great vessels (33). In rats bis-diamine treatment during early rodent embryogenesis (days 9–11 of gestation) causes a high incidence of persistent truncus arteriosus which is frequently accompanied by anomalous development of the aortic arch artery derivatives (34). Aplasia or hypoplasia of the thymus, parathyroid, and thyroid glands occurs frequently following bis-diamine treatment (34).

Isotretinoin, a retinoid prescribed for cystic acne in humans, causes a complex of malformations involving craniofacial, cardiac, thymic, and central nervous structures (35). Although the similarity in these complexes of malformations to those seen following neural crest ablation may be coincidental, it seems more likely that they both involve decreases in the ectomesenchymal population seeding the caudal pharyngeal arches.

CONCLUSIONS

Persistent truncus arteriosus is fairly rare in children with congenital heart disease. However, the other malformations produced by neural crest ablation are much more prevalent in children. It is still not understood why the most common congenital heart malformations in children are not seen more frequently in chick embryos. It may be that neural crest is only a minor contributor for production of heart malformations of this

type. However, the possibility exists that ontogenetic and phylogenetic differences between the cardiovascular systems of avians and mammals result in a different incidence of defects following neural crest injury. For example, the definitive aortic arch in avians is on the right while in humans this is abnormal. In addition, the ductus arteriosus is normally present bilaterally in chick embryos while again this is abnormal in humans.

Many previous investigations of cardiovascular malformation have proposed that dysmorphogenesis results from functional abnormalities which change hemodynamic parameters within the developing system. So far the primary cause of altered hemodynamics has not been addressed. Injury of neural crest-derived ectomesenchyme in the pharyngeal arches provides a potential explanation for altered hemodynamics which might result in a variety of heart malformations.

It is tempting to be satisfied that injury to neural crest cells populating arches 3–6 and the outflow septa constitutes the pathogenesis of persistent truncus arteriosus. If the injury occurs prior to or at the level of the arches then the entire pharyngeal apparatus will be affected which will result in DiGeorge syndrome (3rd and 4th pharyngeal pound syndrome). If injury of the neural crest occurs on a more localized level within the outflow septa, then persistent truncus arteriosus will occur in the absence of any other malformations. Although this may be a major mechanism for pathogenesis of persistent truncus arteriosus, several other elements are important in production of the outflow septa. These are still not well understood. It is hoped that manipulation of the neural crest using microsurgery and selective teratogens will provide more information which will ultimately lead to a complete understanding of normal heart development.

It should be obvious by now that the role of neural crest in heart development is very complex. The neural crest represents only a single cell population which participates in development of the cardiovascular system. It is important that studies of cardiovascular development be pursued using a multidisciplinary approach which includes clinicians and basic scientists with wide-ranging interests and skills.

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