Proteins Associated with Cardiac Neural Crest in the Pharyngeal Region of Early Chick Embryos

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ABSTRACT. Normal septation and alignment of the outflow tract of the heart depends on ectomesenchymal cells derived from the cardiac neural crest. Removal of the premigratory cardiac neural crest results in a variety of cardiac anomalies. The sequence of events leading to these cardiac anomalies in the absence of the cardiac neural crest cell population is not fully understood. This study is the first in a series directed at understanding the role of the neural crest in cardiovascular development at the molecular level. We have begun by distinguishing proteins uniquely expressed by, or responding to the presence of, cardiac neural crest cells in the pharyngeal apparatus, as well as proteins that are seen in the absence of cardiac neural crest cells. Cardiac neural crest was removed by microcautery from stage 9 to 10 embryos. At stages 14, 18, and 21, the protein patterns in the pharyngeal regions of experimental and sham-operated embryos were compared using isoelectric focusing followed by SDS-polyacrylamide gel electrophoresis. Five proteins were found in sham-operated embryos at stages 14 and 18 that could not be seen in embryos with neural crest ablations. Four proteins were found in embryos with neural crest ablation at stages 14 and 18 that were not seen in sham-operated embryos. By stage 21, the protein patterns of both sham-operated and experimental embryos were identical. These results indicate that there may be a compensatory response to neural crest ablation as development proceeds. (*Pediatr Res* 33: 43–47, 1993)

Abbreviations

IEF, isoelectric focusing
DNase, deoxyribonuclease
RNase, ribonuclease

Cells migrating from the neural crest located on the neural axis between the midotic placode and the caudal boundary of somite 3, also known as the cardiac neural crest (Fig. 1), play an important role in the development of the heart and great arteries as well as noncardiovascular structures derived from the pharyngeal apparatus (1–4). In the chick embryo, cardiac neural crest cells migrate shortly after the neural tube has closed. The cells migrating from the cardiac neural crest via the dorsolateral pathway arrest their migration in the circumpharyngeal region before the formation of the pharyngeal arches (5). Cells from the circumpharyngeal crest populate pharyngeal arches 3, 4, and 6 as they are formed. In the pharyngeal arches, the cells provide an ectomesenchymal sheath surrounding each of the aortic arch

arteries (6). These cells provide precursors to nervous tissue and much of the ectomesenchyme to support various aspects of cardiovascular and glandular development in the pharyngeal region. The neural derivatives form the postsynaptic parasympathetic innervation of the heart, thorax, and abdomen, while the ectomesenchymal tissue surrounds the aortic arch arteries to provide support and ultimately develop into the tunica media of the great arteries (7, 8). Some neural crest-derived cells from these arches continue their migration into the cardiac outflow tract where they play a crucial role in forming the outflow septation complex (1, 9).

The ectomesenchymal component of the cardiac neural crest is determined early. Transplantation studies have shown that other areas of the neural crest that contain ectomesenchymal cells are not capable of supporting cardiovascular development (10). Because the transplantation was done at the neural fold stage of development (stage 9), it was inferred that the mesenchymal component of the cranial region of the neural crest is determined early in development. The idea that the neural crest attains a segmental identity early in development is supported by the regional expression of various homeotic messages (11) as well as a rather strict segmental origin of neural crest cells in the cranial region (12).

Normal development of noncardiovascular structures in the pharyngeal apparatus is also dependent upon cardiac neural crest-derived cells in pharyngeal arches 3, 4, and 6 (3, 4). These structures include the thymus, parathyroid, and thyroid glands. The continuity between abnormal septation of the outflow tract and abnormal development of these glands is evident in the clinical entity of DiGeorge syndrome, which is now thought to be a result of abnormal development of the cardiac neural crest (4).

The function of the cardiac neural crest cells is partially compensated when it is bilaterally ablated, by cells derived from the nodose placodes (13, 15). As much as 70% of postsynaptic parasympathetic innervation is present in hearts of chick embryos with bilateral ablation of the cardiac neural crest (14). Ectomesenchymal tissue supporting the aortic arch arteries and cardiac outflow septation is also reconstituted by cells derived from the nodose placodes (15). However, cells derived from the nodose placodes are unable to participate in normal outflow septation (15) or manufacture normal vessel wall components (16). Consequently, outflow tract anomalies occur (15), and the tunica media of the great arteries is delayed in acquiring its elastic matrix (16). The deficient wall support of the aortic arch arteries and other changes in the pharyngeal apparatus could explain the early and significant hemodynamic changes noted in stage 18 chick embryos by Leatherbury *et al.* (17) and Tomita *et al.* (18). Such early hemodynamic changes cannot be explained by failure of outflow tract septation, inasmuch as this septation does not occur until stages 25–27.

The role of the cardiac neural crest cells in the pharyngeal apparatus may be better understood through their molecular activities in this region. Using IEF in conjunction with SDS-

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Fig. 1. Diagram illustrating the extent of the cardiac neural crest on the neural axis.

PAGE, we have discovered the presence of several proteins produced by or in response to cardiac neural crest cells in the pharyngeal apparatus during early stages of development, as well as proteins produced by cells in the absence of cardiac neural crest cells. Differences in proteins produced in these embryos are lost as development proceeds.

MATERIALS AND METHODS

Fertilized Arbor Acre chicken eggs were incubated in forced-draft incubators at 38°C. Relative humidity during incubation was 97%. All eggs were windowed and stained (19) at stages 9–10 of Hamilton and Hamburger (20) and the vitelline membrane torn in preparation for bilateral ablation of the cardiac neural crest by microcautery as described previously (2). In a single day, a total of 16 to 28 embryos were obtained for surgery. Half of the embryos underwent microcautery of the cardiac neural crest (Fig. 1). The other half served as surgical shams that had been subjected, in parallel, to all of the surgical procedures with the exception of microcautery ablation. The eggs were returned to the incubator to allow further maturation of the embryos. Experimental embryos (bilateral cardiac neural crest ablation) and sham-operated embryos (intact cardiac neural crest) were harvested at stages 14, 18, and 21 (18). In each harvest, an equal number of embryos from both experimental and sham-operated groups were dissected by the same individual (M.L.K.) so as to maintain consistency. Dissection was performed with the aid of a stereomicroscope. For the purposes of these experiments, the pharyngeal apparatus was defined to extend from the cranial end of the third pharyngeal arch to the caudal end of the sixth pharyngeal arch region (Fig. 2). This region was separated from the remaining parts of the embryonic head and trunk and the heart, which connects to the pharyngeal apparatus by the cardiac outflow tract. A single pharyngeal apparatus was collected for each gel. A total of at least 20 pharyngeal apparatuses was collected from each stage to be analyzed. In preliminary experiments, it was found that there was not sufficient protein at stage 14 to obtain reproducible protein patterns after electrophoresis, and so in subsequent experiments, the pharyngeal apparatuses were pooled from 10 to 20 embryos at this stage. At stages 18 and 21, a single pharyngeal apparatus was processed to produce a single gel. The tissue was prepared using a slightly modified version of the protocol recommended by Garrels (21). Each pharyngeal apparatus was placed in an Eppendorf tube containing 100 μ L of 1.5 mM Tris buffer (pH 7.4) containing 10 μ g/mL protease-free DNase 1 (Worthington, Enzymes & Biochemicals for Life Science Research, Freehold, NJ), 5 μ g/mL protease-free RNase A (Worthington) and 0.1 mM phenylmethane sulfonyl

Fig. 2. Diagrams of stage 14, 18, and 21 embryos to illustrate the region dissected for analysis by two-dimensional gel electrophoresis. The postotic portion of the pharyngeal region was analyzed. It extends from the cranial border of the third pharyngeal arch to the caudal border of the sixth pharyngeal arch region and was dissected free of the embryo and the heart.

fluoride (Sigma Chemical Co., St. Louis, MO), and homogenized for 20 s on ice using a hand held Omni 1000 tissue homogenizer. Phenylmethane sulfonyl fluoride was used to inhibit the activity of serine type proteases, the most commonly encountered type in blood. Other protease types, although present, are usually identified with specific organ systems. For example, metallo- and cysteine-proteases are usually associated with the digestive system (22). For these experiments, we separated the pharyngeal regions from organs that could contribute these contaminants and interfere with the results. If protease activity were present, one would expect to find an increase in low molecular weight products as well as reduced rather than absent bands. In addition, all experiments were carried out at 4°C under denaturing conditions. Proteases encountered in chick embryos have an optimum temperature of 38°C. RNase and DNase were found to improve the quantity and quality of proteins observed (21, 23). The homogenate was immediately frozen in liquid nitrogen and lyophilized overnight. The dried sample was resuspended in IEF buffer consisting of freshly prepared 9.5 M ultra-pure urea (Bio-Rad Laboratories, Richmond, CA), 2% Nonidet P-40 (Sigma), and 2% ampholyte (LKB Instruments, Inc., Gaithersburg, MD) (23, 24). The protein concentration was measured using the Bradford method (25).

In preliminary experiments, the homogenates of stage 18 embryos were digested for 0–30 min with testicular hyaluronidase (0.1 mg/mL) or chondroitinase ABC (0.1 U/sample) was found in preliminary experiments to digest glycoconjugates completely).

The Bio-Rad Mini-Protean system for IEF-SDS-PAGE was used. First-dimension IEF gels (23) with pI ranges of 3–10 or 5–8 were loaded with 40 μ g of protein in 20–25 μ L of loading buffer. The sample was overlaid with an equal volume of buffer containing 9 M urea, 1% ampholyte with a pH range corresponding to that in the sample buffer and 0.005% Bromophenol blue. Before loading the sample, the first-dimension gels were preelectrophoresed at 200 V for 10 min, 300 V for 15 min, and 400 V for 15 min. After the samples were added, the gels were routinely run at 500 V for 10 min and 750 V for 4 h. IEF was determined

in preliminary experiments to be complete after 4 h. Samples from sham-operated and experimental embryos of each stage were run in pairs to control for environmental variables. When IEF was complete, the gels were extracted from the tubes and equilibrated in denaturing buffer for 2 h. They were attached with 1% agarose in SDS sample buffer to the top of 10% SDS-PAGE slab gels. The SDS-PAGE second dimension included a 10% acrylamide separating gel with a 5% acrylamide stacking gel using a discontinuous buffer system as described by Laemmli (26). Ten percent was determined in preliminary experiments to obtain the best separation of the bulk of proteins in the pharyngeal apparatus. The slab gels were electrophoresed for 0.5–1.5 h at 150 V. Sham-operated and experimental samples were run in parallel. The slab gels were stained twice for demonstration of proteins in low abundance, as recommended by the protocol using a silver kit (Bio-Rad) to visualize proteins.

RESULTS

Viability of the embryos to stages 14 through 21 ranged from 70–90%. The viability of embryos with cardiac neural crest ablations to d 11 of incubation is usually about 30% with an incidence of persistent truncus arteriosus of 80–100%. It has been shown previously that the embryos that survive to d 11 of incubation are a selected population in which the cardiac output is higher than in control embryos (18). The stages chosen for this study represent a time period before this selection process and hence a mixed population of embryos with regard to survival potential.

Although it was possible to visualize the proteins from individual stage 14 pharyngeal regions, the resolution was improved by pooling the pharyngeal samples at this age. The optimum results were obtained when pharyngeal regions of 10 embryos were pooled to obtain sufficient protein (20–40 μ g) at stage 14 for resolution on two-dimensional gels. The pharyngeal regions of individual embryos were used at stages 18 and 21 to generate single two-dimensional gels. Each stage was repeated a minimum of five times in each of the ampholyte focusing gradients (3–10 or 5–8) followed by SDS-PAGE at 7, 10, 12, or 17% acrylamide concentration. Visualization of the proteins that were different was optimized at an ampholyte gradient of 5–7 and SDS-PAGE acrylamide concentration of 10%. Although these parameters restrict the molecular weight and isoelectric points of the proteins analyzed, we found that the proteins outside this range were difficult to compare from gel to gel. Hence, the proteins identified in this study are those that yielded consistent results on the majority of the gels.

Ovalbumin (pI = 6.6, molecular mass = 42 kD) was naturally present in all of the samples and served as an internal standard.

At stage 14, four proteins were found on 2-dimensional gels of samples from the pharyngeal regions of sham-operated embryos that could not be seen on comparable gels of experimental embryos (Table 1, Fig. 2). All four proteins had pI in the 5.5–7.0 range and molecular masses ranging from 42 to 70 kD. One protein (pI = 7.25, molecular mass = 42 kD) was found in gels of the experimental embryos but was not present in sham-operated embryos (Fig. 3, Table 1). Proteins >150 kD or <20 kD were not resolved in this system.

At stage 18, one protein was found in gels of the sham-operated embryos that was not present in experimental embryos (Table 1, Fig. 4). This protein had a pI of 7.0 and a molecular mass of 45 kD. Three proteins that were not present in sham-operated embryos were found on gels of experimental embryos. These proteins had pI ranges of 6.0–7.25 and widely divergent molecular mass (35, 42, and 97 kD). The protein located at pI 7.25 and molecular mass 42 kD had characteristics similar to the same protein found in experimental embryos at stage 14. Digestion of the homogenates before IEF, using testicular hyaluronidase or chondroitinase ABC, resulted in disappearance of this protein, indicating that it is probably a matrix component.

Fig. 3. Two-dimensional gels of stage 14 pharyngeal region. A total of four proteins (1–4) can be seen in sham-operated embryos (a) that are missing in experimental embryos (b). One protein at 42 kD is seen in the experimental embryos but is missing in the sham embryos. *, ovalbumin.

Table 1. Isoelectric points and molecular masses of proteins expressed in the pharyngeal region of sham-operated or experimental embryos at stages 14 and 18 after cardiac neural crest ablation

Protein	pI	Molecular mass (kD)	Sham/experimental
Stage 14			
1	6.5	70	+/-
2	6.5	50	+/-
3	7.0	50	+/-
4	5.5	42	+/-
5*	7.25	42	-/+
Stage 18			
1	7.0	25	+/-
2	6.5	35	-/+
3*	7.25	42	-/+
4	6.75	97	-/+

* Protein digested with hyaluronidase and chondroitinase ABC.

By stage 21, the protein patterns of samples from experimental and sham-operated embryos were strikingly similar (Fig. 5). No consistent differences could be found on gels from embryos at this stage. The protein found in stage-14 and -18 experimental embryos had disappeared.

DISCUSSION

This study has shown five proteins that are uniquely associated with the presence of cardiac neural crest cells in the pharyngeal apparatus at stages 14 and 18 of chick development. Stage 14 is approximately 48 h of development, whereas stage 18 is approximately 72 h of development. Hence, the separation in time between these two stages is only about 24 h. Even so, the protein patterns of the pharyngeal apparatus at these two different stages are quite different. The proteins that were seen in gels of embryos with an intact neural crest may be produced by the neural crest cells themselves or by some other cell type located in the pharyngeal region in response to the presence of the cardiac neural crest cells. Four (or possibly three) additional proteins are associated

Fig. 4. Two-dimensional gels of stage 18 pharyngeal region. One protein (1) that is missing from the experimental embryo (b) can be seen in the sham-operated embryo (a). Three proteins are seen in the experimental embryo that are not present in the sham-operated embryo. Note that protein 3 in (b) is in the same position as protein 5 in Figure 3b. *, ovalbumin.

Fig. 5. Two-dimensional gels of stage 21 pharyngeal region. The protein patterns of sham-operated (a) and experimental embryos (b) are the same. *, ovalbumin.

with the absence of cardiac neural crest cells in the pharyngeal region. These proteins may be produced in response to the lack of neural crest cells or they may not be normally degraded or transformed in the absence of the neural crest.

The similarity of protein patterns in stage-21 chick embryos with and without cardiac neural crest ablation might be interpreted to mean that the unique role of cardiac neural crest cells in this region is completed after stage 18 and before stage 21. On the other hand, it could also indicate that the stage 21 embryo has compensated for the loss of the cardiac neural crest cell

population. These interpretations are consistent with the morphologic events occurring in the pharyngeal apparatus, which include the population of pharyngeal arches 3–6 during the time period between stages 14 and 18 (5, 9). By stage 21, the neural crest cells have fully populated these arches, and migration has been initiated into the outflow tract of the developing heart where they will participate in forming the outflow septation complex. In the present study, the heart and outflow tract were not included in the tissue sampled in order to analyze the pharyngeal compartment without the added complexity of cardiac-associated proteins.

The finding that the pharyngeal protein patterns at stage 21 are similar in sham-operated and experimental embryos does not preclude other changes in the normal pattern of development as the cells migrate into other more distal targets, *e.g.* the outflow region of the heart.

The protein at pI 7.25 and molecular mass 42 kD, found in experimental embryos at stages 14 and 18 but disappearing by stage 21 is most likely associated with the matrix. This protein is found in the same position in IEF-SDS-PAGE gels of heart at the same stages of development (unpublished data). The relatively large quantity of this protein and its behavior on treatment with chondroitinase lead to the assumption that it is one of the glycoconjugate components of the cardiac jelly (28, 29). Its presence in the pharyngeal region in experimental embryos, but not in sham-operated embryos, is puzzling but perhaps represents displacement of cardiac jelly as a spatial surrogate for the missing neural crest cells.

Any protein uniquely expressed in the pharyngeal apparatus of chick embryos with intact cardiac neural crest may be hypothetically associated with a number of developmental events related to cardiovascular development, such as normal development of the supportive tissue of the aortic arch arteries and cardiac outflow tract. The significance of this may be substantial, inasmuch as the hemodynamic changes noted at earlier stages of development of chick embryos with ablated cardiac neural crest (17, 18) may be a result of the lack of normal vascular wall support, which may have a direct or indirect effect on the process of cardiac outflow septation and alignment. It might initiate a cascade of events responsible for development of cardiovascular structures in the pharyngeal apparatus and upstream to the outflow tract and heart through the endothelial cell column or bloodstream. In preliminary studies using digestions of the tissue homogenates with testicular hyaluronidase and chondroitinase ABC prior to IEF, we found several proteins that were shifted following digestion. Only the one mentioned above disappeared following the digestion in comparing the protein patterns in experimental *versus* sham-operated embryos. This indicates that the proteins associated with the presence of the neural crest are probably not incorporated in the glycoconjugates of the extracellular matrix.

Since development of the normal glandular derivatives of the pharyngeal apparatus depends on an intact neural crest (1, 3), any of these proteins could be involved in development of noncardiovascular structures in the pharyngeal apparatus. This includes thymus, thyroid, and parathyroid glands (3, 4).

The remaining proteins expressed in the absence of neural crest cells may be normally suppressed or transformed by cardiac neural crest cells, or they may be produced as a compensatory response to their absence. In regard to the latter possibility, it has been shown that cells derived from the nodose placodes reconstitute neural and some ectomesenchymal cells that participate in cardiovascular development when the cardiac neural crest is removed (13, 15). Interestingly, cells from the nodose placodes begin their migration at stage 14 (30), an age at which we identified only one atypical protein in the pharyngeal apparatus of experimental embryos. By stage 18, when three atypical proteins are found in the experimental pharyngeal region, cells derived from the nodose placodes are well established in the pharyngeal region (30).

A second possibility is that, under normal conditions of development, these atypical proteins are suppressed by the cardiac neural crest cells as they populate the pharyngeal apparatus. There is some evidence that the neural crest cells can alter their migratory pathway as they migrate through an area. Unfortunately, we have not been able to obtain reliable results using IEF-SDS-PAGE in the pharyngeal region of embryos younger than stage 14.

There is an additional possibility that some of the proteins expressed in the experimental embryos are heat-shock proteins that are induced in response to the heat of cauterization. However, only one protein is found in experimental embryos at stage 14 (24 h after the cauterization), whereas three are found at stage 18 (48 h after cauterization). This time course of activation does not fit the usual pattern of heat-shock proteins which are expressed transiently within a few hours of the heat stress (32). It might be possible that some of the proteins at stage 18 are heat-shock proteins that are expressed in response to hemodynamic stress caused by neural crest absence. It is well documented that hemodynamic stress can induce the expression of heat-shock proteins (32), and it is also well documented that neural crest ablation results in hemodynamic changes that can be measured at stage 18 (17, 18).

The role of these proteins in the pharyngeal apparatus can only be defined after sequencing and classification of the proteins. It will be essential to generate MAb or nucleotide probes to determine which cell types these proteins are associated with in the pharyngeal region.

This is a preliminary report in a continuing study to elucidate the role of the cardiac neural crest cells in the pharyngeal apparatus at the molecular level. Identification of proteins in the pharyngeal apparatus expressed either by or in response to cardiac neural crest cells will ultimately lead to a better understanding of the role and mechanism of action of this cell population in cardiovascular development.

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