Peripheral development of the avian vagus nerve with special reference to the morphological innervation of heart and lung

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Summary. The development of the cardio-pulmonary innervation was studied in whole-mount specimens of chick embryos stained with the anti-neurofilament protein (NFP) antibody. From the morphological point of view, vagal branches could be classified into two categories, i.e., the branchial branches primarily related to the pharyngeal arches, and intestinal arborization derivatives which are associated primarily with the primitive gut. The former consisted of the superior cardiac branch innervating the truncus arteriosus of the heart, and the latter, the sinal branch, pulmonary branches as well as recurrent nerve and the other intestinal branches. The superior cardiac branch at first develops as a pair of branchial branches which passes into the truncus arteriosus at stage 25, and later rotates along the aortic arch 6, thus making an asymmetrical configuration by stage 27. The sinal branch is a medial branch which first develops at stage 24. It arises from the junction of each intestinal arborization in close association with the pulmonary branch.

Key words: Chick embryos – Vagus nerve – Pharynx – Cardiopulmonary innervation

Introduction

The vagus nerve consists of two components, i.e., the branchial branches which are related to the pharyngeal arch system, and the intestinal portion associated with the intestinal tract. The branchial portion of the nerve has been regarded as derived from several segments of nerve roots which have been collected into a single root (Johnston 1905). The segmental pattern of innervation in pharyngeal arches 3 to 6 has become less clear in higher vertebrates than that in the shark (Tanaka and Nakao 1979). This is probably because of the modification of the posterior portion of the pharynx. The intestinal portion of the vagus, on the other hand, consists of a widespread nerve network innervating the gut and shows no clear branches.

Among the vagal innervation, the cardio-pulmonary branches have usually been studied with great interest, since the discovery of the depressor nerve in the heart (Cyon and Ludwig 1867). Autonomic innervation of the heart and the origin of cardiac ganglia have been studied in the chick embryo (Rickenbacher and Müller 1979; Kirby et al. 1980; Kirby and Stewart 1983; Kirby 1988). It is significant that a portion of the vagal neural crest, “cardiac neural crest” contributes both to cardiac ganglia and to the morphogenesis of the heart itself (reviewed by Kirby 1987). This finding is extremely important in the comparative morphology and embryology of the heart, because it shows clearly that the development of the heart, including cardiac ganglia, is closely associated with the pharyngeal arch system, whose derivatives are mostly provided by the cranial neural crest (reviewed by Le Douarin 1982). Sensory innervation of the heart has been found in the chick to come from the nodose ganglion of the vagus nerve (Walkey and Bower 1981). This ganglion originates from one of the epibranchial placodes, the nodose placode (Narayan and Narayan 1980; D'Amico-Martel and Noden 1983). The morphology of vagal branches has been worked out in several vertebrate species or their embryos (His 1891; Shaner 1930; Hirakow and Miki 1971). As noted above, the findings of these authors have to be reconsidered in terms of the embryology of the pharyngeal arch system, especially of the vagal neural crest migration. In spite of such anatomical studies of vertebrate embryos, complexities still remain in the terminology of the nervous system in this region, as mentioned by several authors (Mizeres 1963; Fukuyama 1982).

The comparative anatomy and developmental morphology of the vagus nerve indicates that the vagal branch along the truncus arteriosus represents the branchial portion of this nerve (Hirakow and Miki 1971). However, the morphological evaluation of the sinal branch (the vagal branch into the sinus venosus, sinoatrial cardiac plexus of Licata 1954), and the pulmonary branches is ambiguous. To clarify these ambiguities, we applied the whole-mount staining technique of the ner-
vus system (Ishikawa et al. 1986) to chick embryos at stage 21–34 (Hamburger and Hamilton 1951) and visualized the developing vagus nerve branches. The vascular system was also observed by injecting ink into the omphalomesenteric vein. In a previous paper on the chick glossopharyngeal nerve using the same methods, we reported on the proximal portion of the vagus nerve and the development of the dorsal pharyngeal branches (Kuratani 1990). The present work is a sequel to the previous work, and is intended to evaluate the morphological significance of vagal branches in the cardio-pulmonary region of chicks by following their development.

**Materials and methods**

**Antibodies.** Commercially purchased monoclonal anti-all NFP (IT-0168, all neurofilaments, 70K + 160K + 210K, DP5 + 43 + 12, Cosmo Bio Co., Ltd.) was used as the primary antibody. As the secondary antibody, peroxidase-conjugated sheep anti-mouse IgG (3611-3244, Cappel) was used.

**Whole-mount specimens.** Eggs of the White Leghorn hen (Gallus domesticus) were incubated in a hatching chamber at 37°C in a humid atmosphere. The embryos were excised from the eggs and fixed in Zamboni’s fixative solution at 4°C for 4 h. Embryonic stages were determined according to Hamburger and Hamilton (1951). After rinsing in phosphate buffered saline (PBS), they were immunohistochemically stained as whole-mounts according to the method of Ishikawa et al. (1986) which has been slightly modified to improve resolution.

The embryos were treated with 1% periodic acid/distilled water for 30 to 45 min. Of the embryos were then incubated 1 to 2 days in freshly prepared 2% Triton X-100, 2% saponin and 8% sucrose/PBS, and they were frozen and thawed twice. In another series, the embryos were incubated with 0.02% collagenase in PBS for 2 h at 38°C. They were refixed with Zamboni’s fixative for 10 min and washed in PBS containing 0.3% Triton X-100 (PBST) until they lost their yellowish color. After washing in PBST, both groups of embryos were incubated with the primary antibody diluted 1/100 in PBST containing 0.2% bovine serum albumin (PBST-BSA) and 0.1% sodium azide for 2 to 4 days at 4°C. They were then washed in PBST for 6 h, and incubated with the secondary antibody diluted 1/100 in PBST-BSA at 4°C for 2 days. After washing in PBST, the embryos were pre-incubated in 40 ml of Tris-HCl buffer with 2 mg 3,3′-diaminobenzidine for 1 h at room temperature. The peroxidase reaction was performed in the same buffer containing 10 μl of 30% hydrogen peroxide at 0°C. Stained embryos were transferred to a graded series of glycerol dilutions and stored in 80% glycerol.

Prior to fixation, some of the specimens were injected with blue ink, or diluted white water paint, into the omphalomesenteric vein, in order to show the vascular system simultaneously. In other cases, the embryos were sliced transversely with a blade, so that better observation of the internal structures would be obtained. These techniques were arbitrarily combined. Thus, several specimens were prepared in each developmental stage. It was also possible to re-stain the stained embryo when necessary, starting from the PBST wash prior to the primary antibody incubation. The specimens were photographed with a Nikon SMZ-10 stereomicroscope equipped with a Nikon UF-X-2 photographic attachment. Observations were performed under the microscope, and developing nerve branches and vessels were drawn on the photograph. The descriptions by Watanabe (1960) and Licata (1954) were referred to for nomenclature.

**Sectioned specimens.** The fixed embryos were washed in PBS, dehydrated in a graded series of alcohol and embedded in paraffin. The sections were cut transversely to the long axis of the trunk. After the removal of paraffin in xylene, the sections were treated in 1% periodic acid for 10 min and washed 3 times in PBS for 10 min each. The sections were then incubated in primary antibody overnight in 4°C, and in secondary antibody for 2 h at room temperature. The peroxidase reaction was performed in the 40 ml Tris-HCl buffer with 2 μg 3,3′-diaminobenzidine, 10 μl of 30% hydrogen peroxide for 45 min at room temperature.

**Results**

**Stage 21**

At this stage, the vagus nerve largely consisted of the following portions, i.e., the ganglionic crest for the

**Fig. 1. Stage 21 chick embryo.** Illustrated from a whole-mount stained specimen. The proximal trunk and the node of ganglion of the vagus nerve at this stage lie lateral to the anterior cardinal vein (vca). Note that a fine branch is growing from the nodose ganglion (gln) within the fourth pharyngeal arch (arrowheads). A communication between the intestinal arborization (ia) and the hypoglossal nerve (XII) is also present (asterisk). Ao3,4, aortic arches; comX, communicating branch of the vagus or the later r. descendens of the vagus; ct, chorda tympani; gc, ganglionic crest of the cranial nerves IX and X; rling, lingual nerve of the glossopharyngeus; v3, mandibular nerve; V–XII, cranial nerves.