Confocal Laser Scanning Microscopy on the Distribution of Neural Nitric Oxide Synthase (nNOS)-immunoreactive Nerves in the Chicken Harderian Gland

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Nitrergic innervation of the chicken Harderian gland was investigated by using immunohistochemistry for neural nitric oxide synthase (nNOS) and a confocal laser scanning microscope. Immunoreactivity for nNOS was detected on nerve elements in the pterygopalatine ganglion and the Harderian gland. Most nerve cells in the pterygopalatine ganglion showed nNOS immunoreactivity. These cells had a variety of sizes and shapes. Nerve fibers projecting from these ganglion cells also showed immunoreactivity for nNOS. Dense networks of nerve fibers showing nNOS immunoreactivity were observed in the interstitial tissue of the Harderian gland. Fine immunoreactive nerve fibers were found running in the capsule. No immunoreactive nerve fibers were found contacting with epithelial cells of the collecting duct. The present data suggested that nitrergic nerves have a relation to the regulation of the immune function of the chicken Harderian gland.

Key words : chicken, confocal laser scanning microscope, Harderian gland, immunohistochemistry, nNOS

Introduction

The chicken Harderian gland is the dominant orbital gland. The gland is a compound tubulo-acinar structure, and its secretory duct opens into the inferomedial portion of the conjunctival sac deep into the nictitating membrane (Wight *et al.*, 1971 a ; Burns, 1976 a). That has two important functions : production of a secretion that lubricates the nictitating membrane (Wight *et al.*, 1971 b), and protection of the oculonasal region from exogenous antigens (Burns, 1976b ; Oláh *et al.*, 1996). The aggregation of plasma cells is observed in the interstitial tissue of the chicken Harderian gland (Mueller *et al.*, 1971 ; Albini *et al.*, 1974). The cells are rich in immunoglobulins, Ig A, Ig G and Ig M (Albini *et al.*, 1974 ; Ohshima and Hiramatsu, 2002). Thus the chicken Harderian gland may contribute as an important component to the local immune system in the oculonasal region (Mueller *et al.*, 1971).

A rich supply of autonomic nerves to the Harderian gland has been recognized in the domestic fowl. Histochemical studies showed the innervation of the gland by catecholamine-containing and acetylcholinesterase (AChE)-positive nerves (Fourman

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and Ballantyne, 1967 ; Walcott *et al.*, 1984 ; Walcott and McLean, 1985). AChE-positive nerves from the pterygopalatine ganglion (Gienc and Zaborek, 1984 ; Walcott *et al.*, 1989) were distributed throughout the gland, especially in the interstitial tissue. Our previous study using AChE histochemistry at electron microscopic level confirmed that AChE-positive nerve terminals were in contact with plasma cells in the interstitial tissue (Hiramatsu and Ohshima, 1999). Immunohistochemistry for neuropeptides (PACAP27, PACAP38 and galanin) have shown that the gland received nerve fibers containing these neuropeptides from the pterygopalatine ganglion (Hiramatsu *et al.*, 1999b).

Nitric oxide (NO), a free radical gas, is converted from L-arginine by the enzyme, nitric oxide synthase (NOS), and considered as one of the neurotransmitters in the mammalian central and peripheral nervous system (Bredt and Snyder, 1992). Niterergic nerves are widely distributed in the chicken peripheral tissue such as pancreas and cecum (Hiramatsu and Ohshima, 1994 ; Hiramatsu *et al.*, 1999a). Little is, however, known about nitergic innervation of chicken lymphoid organs. The existence of neural NOS (nNOS)-immunoreactive nerves was reported in the bursa of Fabricius and the thymus of the chicken (Ali *et al.*, 1996 ; Gulati *et al.*, 1997). This study aimed at clarifying the distributional pattern of nNOS-immunoreactive nerves in the chicken Harderian gland, the local immune tissue in the oculonasal region, using a confocal laser scanning microscope. The involvement of nitergic nerves in the local immune system was also discussed in this paper.

Material and Methods

Adult male White Leghorn chickens (body weight 1.8–2.3 kg, n=8) were used in this study. They were kept in our laboratory under controlled light condition (12 h light : 12 h darkness) and given a commercial diet and water *ad libitum*. Chickens were perfused with periodate-lysine-paraformaldehyde (PLP) fluid following 0.75% sodium chloride solution under the anesthesia with sodium pentobarbital. Harderian glands were immediately removed from an orbital cave of both sides and each gland was cut into two tissue blocks with a razor blade. Tissue blocks were immersed in the same fresh fixative at 4°C for 6 hours and transferred into 20% phosphate-buffered sucrose for the cryoprotection. And then frozen sections at 14µm thickness were made in a cryostat in the conventional manner.

Indirect immunofluorescent technique was applied to detect nNOS immunoreactivity. Frozen sections treated with 10% normal goat serum were incubated with rabbit anti-porcine nNOS serum (Alexis, USA, 210-501-R025, diluted to 1 : 500) at room temperature overnight. After several washing with phosphate buffered saline (PBS), they were incubated with FITC conjugated goat anti-rabbit IgG (EY Laboratories, USA, FA-2307-2, diluted to 1 : 100). Sections were coverslipped with Fluoromount-GTM (Southern Biotechnology Associates, USA) after several washing with PBS. Sections were observed and photographed under a confocal laser scanning microscope (Fluoview, Olympus, Japan).

Normal rabbit serum or the omission of the primary antiserum followed by the

secondary antiserum was used as the negative controls. Immunoreactivity was not observed in either case.

Chickens were treated in accordance with the "Guideline for Regulation of Animal Experimentation (1997)" of Faculty of Agriculture, Shinshu University.

Results

Immunoreactivity for nNOS was observed on the nerve elements in the pterygopalatine ganglion and the Harderian gland.

Many nerve cells in the pterygopalatine ganglion showed immunoreactivity for nNOS (Fig. 1 a). Immunoreactive nerve cells had a variety of sizes and shapes (Fig. 1 b). The initial segments of nNOS-immunoreactive nerve cells also showed strong immunoreactivity (Fig. 1 b, arrow). Nerve fibers showing nNOS immunoreactivity were running in the ganglion. Nerve bundle containing many fine nerve fibers showing nNOS immunoreactivity was found along with the Harderian gland (Fig. 2).

In the parenchyma of the gland, nNOS-immunoreactive nerve fibers were mainly distributed in the interstitial tissue (Figs. 3 and 4). They made dense networks in the interstitial tissue beneath the epithelium of the collecting duct. Fine nerve fibers showing nNOS immunoreactivity were observed in the capsule and but not in the exocrine region of the gland (Figs. 3 and 5). They were also found in the adventitia of blood vessels along with the Harderian gland (Fig.5).

Fig. 1. nNOS-immunoreactive nerve cells in the pterygopalatine ganglion. a) Many nNOS-immunoreactive nerve cells are observed in the large ganglion (G) along with the Harderian gland (HG). b) High-powered view of Fig. 1 a. nNOS-immunoreactive nerve cells show a variety of sizes and shapes. Arrow indicates the initial segment of a ganglion cell showing nNOS immunoreactivity.

Fig. 2. Nerve bundle (arrow) along with the dorsal edge of the Harderian gland (HG) contains many nerve fibers showing nNOS immunoreactivity.

Fig. 3. nNOS-immunoreactive nerve fibers in the parenchyma of the Harderian gland. Immunoreactive nerve fibers are found in the interstitial tissue, but not in the exocrine region beneath the capsule. Arrow indicates fine nerve fiber in the capsule. PD : primary duct.

Fig. 4. High-powered view of the interstitial tissue of the Harderian gland. nNOS-immunoreactive nerve fibers form a dense network in the interstitial tissue beneath the epithelium of the primary duct (PD).

Fig. 5. Fine nerve fibers showing nNOS immunoreactivity were observed in the capsule of the gland (arrows) and the adventitia of the blood vessel (B). No nerve fibers showing nNOS immunoreactivity are found in the exocrine region (E) of the gland.

Discussion

The chicken Harderian gland is considered as the local immune tissue at the oculonasal region, since numerous plasma cells are accumulated in its interstitial tissue (Mueller *et al.*, 1971 ; Burns, 1975 ; Niedorf and Wolters, 1978) and immunoglobulins are produced and secreted in response to the local antigen stimulus (Albini *et al.*, 1974 ; Burns, 1976 b ; Oláh *et al.*, 1996). The present study clarified the distributional pattern of nNOS-immunoreactive, nitrergic, nerves in the gland. Nerve fibers immunoreactive for nNOS were distributed in the interstitial tissue of the gland and made dense network. Many studies have shown the interactions between the nervous system and the immune system. Enzyme histochemistry for AChE and immunohistochemistry for neuropeptides by co-workers and myself suggest the important role of cholinergic and peptidergic nerves in the local immunity by the chicken Harderian gland (Hiramatsu and Ohshima, 1999 ; Hiramatsu *et al.*, 1999 b). We have demonstrated the terminals of AChE-positive nerves on plasma cells in the interstitial tissue of the chicken Harderian gland (Hiramatsu and Ohshima, 1999). A few studies have shown the nitrergic innervation of the immune organs in the chicken. Ali *et al.* (1996) indicated the close association of nitrergic fibers with lymphatic nodules of the bursa of Fabricius of the chicken. Gulati *et al.* (1997) showed the existence of nitrergic nerves in the chicken thymus. Nitrergic nerve fibers were also found in the cecal tonsil of the chicken (Hiramatsu *et al.*, 1999 a). These findings suggest the participation of the nitrergic nerves in the immune response. In the present study dense networks of nNOS-immunoreactive nerves were observed in the interstitial tissue. This finding implies a close relationship between nitrergic nerves and plasma cells. So it is likely that nitrergic nerves in the chicken Harderian gland play an important role in the regulation of plasma cells.

Many nerve cells in the pterygopalatine ganglion showed immunoreactivity for NOS. This ganglion also contained a lot of AChE-positive cells and several types of neuropeptide-containing cells (Hiramatsu and Ohshima, 1999 ; Hiramatsu *et al.*, 1999 b). These data suggest that NO coexists with other neurotransmitters, acetylcholine and neuropeptides. Cholinergic, peptidergic and nitrergic nerves projecting from the pterygopalatine ganglion may co-work in the regulation of the local immune system by the Harderian gland.

References

- Albini B, Wick G, Rose E and Orlans E. Immunoglobulin production in chicken Harderian glands. *International Archives of Allergy*, 47 : 23-34. 1974.
- Ali SM, Chan AS and Leong SK. Histochemical and immunohistochemical localization of nitrergic neural and non-neural cells in the bursa of Fabricius of the chicken. *Cell and Tissue Research*, 285 : 273-279. 1996.
- Bredt DS and Snyder SH. Nitric oxide, a novel neuronal messenger. *Neuron*, 8 : 3-11. 1992.
- Burns RB. Plasma cells in the avian Harderian gland and the morphology of the gland in the rook. *Canadian Journal of Zoology*, 53 : 1258-1269. 1975.
- Burns RB. The structure of the lachrymal glands of the domestic fowl and of the duck. *Research in Veterinary Science*, 21 : 292-299. 1976 a.

- Burns RB. Specific antibody production against a soluble antigen in the Harderian gland of the domestic fowl. *Clinical and Experimental Immunology*, 26 : 371–374. 1976 b.
- Fourman J and Ballantyne B. Cholinesterase activity in the Harderian gland of *Anas domestica*. *Anatomical Record*, 159 : 17–28. 1967.
- Gienc J and Zaborek E. The pterygopalatine ganglion of hen. *Folia Morphology (Warsz)*, 43 : 191–195. 1984.
- Gulati P, Tay SS and Leong SK. Nitergic, peptidergic and substance P innervation of the chick thymus. *Journal of Hirnforschung*, 38 : 553–564. 1997.
- Hiramatsu K and Ohshima K. Colocalization of NADPH-diaphorase with neuropeptides in the intrapancreatic neurons of the chicken. *Neuroscience Letters*, 182 : 37–40. 1994.
- Hiramatsu K and Ohshima K. Ultrastructural localization of acetylcholinesterase (AChE) activity in the chicken Harderian gland. *Histology and Histopathology*, 14 : 445–451. 1999.
- Hiramatsu K, Kawamori Y and Ohshima K. Histochemical study on the distribution of nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd)-positive neurons in the chicken caecum. *Anatomia Histologia Embryologia*, 28 : 345–349. 1999 a.
- Hiramatsu K, Tanaka N, Nakamura N and Ohshima K. Immunohistochemical study on the innervation of the chicken Harderian gland by peptides (galanin, PACAP27 and PACAP38)-containing nerves. *Japanese Poultry Science*, 36 : 19–24. 1999 b.
- Mueller AP, Sato K, Glick B. The chicken lacrimal gland, gland of Harder, caecal tonsil, and accessory spleens as sources of antibody-producing cells. *Cellular Immunology*, 2 : 140–152. 1971.
- Niedorf HR and Wolters B. Development of the Harderian gland in the chicken : light and electron microscopic investigations. *Investigation of Cell Pathology*, 1 : 205–215. 1978.
- Ohshima K and Hiramatsu K. Immunohistochemical localization of three different immunoglobulin classes in the Harderian gland of young chickens. *Tissue and Cell*, 34 : 129–133. 2002.
- Oláh I, Kupper A and Kittner Z. The lymphoid substance of the chicken's Harderian gland is organized in two histologically distinct compartments. *Microscopy Research and Technique*, 34 : 166–176. 1996.
- Walcott B, Keyser KT, Sibony P and Karten HJ. The histochemistry of the pterygopalatine ganglion and Harderian gland of the chicken. *Society of Neuroscience Abstract*, 10 : 434. 1984.
- Walcott B and McLean JR. Catecholamine-containing neurons and lymphoid cells in a lacrimal gland of the pigeon. *Brain Research*, 328 : 129–137. 1985.
- Walcott B, Sibony PA and Keyser KT. Neuropeptides and the innervation of the avian lacrimal gland. *Investigative Ophthalmology & Visual Science*, 30. 1666–1674. 1989.
- Wight PAL, Burns RB, Rothwell B and MacKenzie GM. The Harderian gland of the domestic fowl. I. Histology, with reference to the genesis of plasma cells and Russell bodies. *Journal of Anatomy*, 110 : 307–315. 1971 a.
- Wight PAL, MacKenzie GM, Rothwell B and Burns RB. The Harderian glands of the domestic fowl. II. Histochemistry. *Journal of Anatomy*, 110 : 323–333. 1971 b.