Nerve innervations in the human adult and fetal parotid duct

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SUMMARY

The human adult parotid duct is the longest of all major salivary gland ducts, approximately 6-8 cm in length. Its unique structure extends over the masseter muscle, penetrates through the buccinator muscle and opens into the oral cavity. Salivary secretion is under basic control of the sympathetic and parasympathetic divisions of the autonomic nervous system. Scarce reporting on the parotid duct nerve distribution led us to this study; to investigate the nervous distribution in the human adult and fetal parotid ducts using an antibody against protein gene product 9.5 (PGP9.5), a molecular marker for nerve cells and fibers. In order to identify the nerve fibers distributed throughout the parotid duct and confirm them to be part of the autonomic nervous system, we stained adult parotid ducts with tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT) for observation. PGP9.5 staining of the parotid duct's inside wall where it traverses over the masseter prior to penetrating the buccinator revealed a dense concentration of nerve fibers in the area. Staining revealed both sympathetic and parasympathetic nerve fibers in the same area, with the majority of the sympathetic nerve fibers surrounding blood vessels. However, the section of the duct penetrating the buccinator showed less concentration of nerve fibers in both adult and fetal specimens. The difference in the nerve distribution of the parotid duct suggests its direct association with the salivary transport function of the duct. PGP9.5 expression in fetuses over five months of age further suggests that the nerve distribution in the human parotid duct is fully established at six months of gestation.

INTRODUCTION

Salivary glands are innervated by postganglionic nerve fibers of the sympathetic and parasympathetic divisions of the autonomic nervous system. Salivary secretion is controlled by autonomic nerves distributed throughout the salivary gland and intraglandular ducts (Avery, 2001; Antonio, 2003; Junqueira and Carneiro, 2003; Ross et al., 2003). It is known that the viscosity of saliva varies based on nerve system's actions. Carpenter reported that "the rate of IgA secretion into saliva is regulated by the autonomic nerves supplying the glands in vivo (Carpenter et al., 2004). Many studies have reported regarding salivary gland innervation using animal experiments (Thulin, 1976; Proto et al., 1981; Ekströ, 1989; Garrett and Kidd, 1993; Tsuboi et al., 2004) and human salivary gland nerve distribution (Heym et al., 1994; Matsuda et al., 1997); however, there are very few detailed reports observing the nerve distribution of the human parotid duct.

PGP9.5 is a neuron-specific protein originally discovered by high-resolution two-dimensional mapping of soluble protein from different human organs (Jackson and Thompson, 1981; Doran et l., 1983) and was found to be the best immunohist-

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chemical marker for the human peripheral nervous system (Thompson et al., 1983; Lauria et al., 2004). Most reports on the salivary gland are based on observation of the nerve distribution in other animal salivary glands. PGP9.5 is considered the most reliable stain for all animals, particularly humans. Because PGP9.5 stains exceptionally well in humans, its use in research is very common, some of which on the human salivary gland. Konttinen used PGP9.5 staining to observe nerve fibers of the labial salivary gland in adult human patients with Sjögren Syndrome (Konttinen et al., 1992). There are generally many nerves distributed in the glandular system as well as in the salivary gland. Wang also used PGP9.5 to observe skin nerve fibers and cells in various areas of the human body (Wang et al., 1990). He reported that there was an abundance of PGP9.5 positive nerves distributed around the acini of eccrin sweat gland.

The role of the parotid duct has commonly been known as a mere passageway for saliva into the oral cavity. We have been studying the parotid duct closely, suggesting that this long duct actually functions as a sphincter, together with the contraction of the buccinator muscle, and regulates the salivary flow into the oral cavity (Amano et al., 2010; 2013). Studying the nerve distribution of this area has a significant impact on the discovery of new findings regarding the unique structure and multifarious functions of the parotid duct. In this study, we conducted PGP9.5 antibody staining of human adult and fetal parotid ducts to observe nerve distribution, and immunohistochemistry staining using TH and ChAT antibodies to identify whether the nerves are from the sympathetic or parasympathetic nervous system of the adult human parotid duct.

MATERIALS AND METHODS

Parotid ducts were obtained from a total of 15 human adult cadavers 55 to 89 years of age and ten fetuses from the cadaver collection of the Department of anatomy, Kyorin University School of medicine, Japan. The fetuses consisted of the following months of gestation: five months x 2, six months x 3, seven months x 2, and nine months x 3. Their gestational ages were determined by measuring their Crown-Rump lengths (CRL) and by using the "Classification of Shimamura" (Shimamura, 1957), a chart to convert CRL to its corresponding age in terms of months of gestation specifically for Japanese fetuses.

After removing the facial skin, a section of the parotid duct from buccinator muscle to the opening into the oral cavity was removed en bloc. Adult specimens were cut into three parts: 1) part of the duct closest to the parotid gland, 2) part of the duct traversing over the masseter, and 3) part of parotid duct penetrating the buccinator. Fetal specimens were too small to cut into smaller sections. All specimens were immediately fixed with 4% para-formaldehyde and 0.01mol phosphate-buffered saline (PBS) solution of with a pH level of 7.2 - 7.4 over 24 hours. After fixation, the specimens were rinsed in PBS with 20% -30% sucrose solutions for at least 24 hours. Further, all parts were sliced into cryostat longitudinal sections of 20μm thickness and thawed on gelatin–coated slides. Adult specimens were immune-stained with PGP9.5, ChAT and TH; fetal specimens were stained only with PGP9.5 in order to observe the basic nerve distribution from a developmental perspective. All observations were conducted and photography taken with the fluorescence microscope digital camera (Keyence, BZ-9000, Osaka, Japan).

All sections were immune-stained with rabbit anti-human PGP9.5 (1:70, Ultraclone, RA 95101) overnight at 4°C, washed in PBS, and rinsed with secondary antibody for 2 hours at 37°C in FITC-conjugated goat anti rabbit IgG (1:400 Boehringer Mannheim, FRG), washed in PBS, and mounted on Vectashield (Vector Inc. Burlingame, CA). For identification of parasympathetic nerves, the adult sections were stained with anti-ChAT polyclonal antibody (1:100 Millipore, CA) overnight at 4°C, washed in PBS, and rinsed with secondary antibody for 2 hours at 37° in FITC-conjugated goat anti rabbit IgG (1:400, Abcam), washed in PBS, and mounted on Vectashiel (Vector Inc. Burlingame, CA). To localize the sympathetic nerves, the adult sections were further stained with TH polyclonal antibody (1:100 Millipore, CA) over night at 4°C, washed in PBS, and rinsed with secondary antibody for 2 hours at 37°C in Cy3-conjugated goat anti-rabbit IgG (1:400, Abcam), washed in PBS, and mounted on Vectashield (Vector Inc. Burlingame, CA).

This study was carried out according to the Declaration of Helsinki's principles for biomedical research and ethical criteria approved by the Institutional Review Board at Kyorin University, Tokyo, Japan.

RESULTS

Expression of protein gene product (PGP) 9.5
Nerve fibers were clearly expressed in all fetuses (five, six, seven, and nine) as early as five months of gestation (Fig. 1A). Furthermore, in all fetuses (Figure 1B), we observed a small concentration of nerve fibers in the section of the parotid duct penetrating the buccinator (Fig. 1Ba). In comparison, we observed a heavier concentration of nerve fibers in the section of the parotid duct crossing over the masseter before entering the buccinator (Figs. 1Bb, 1Bc). Likewise, in all adult specimens, nerve fibers were abundant in the area close to the parotid gland where it crosses over the masseter
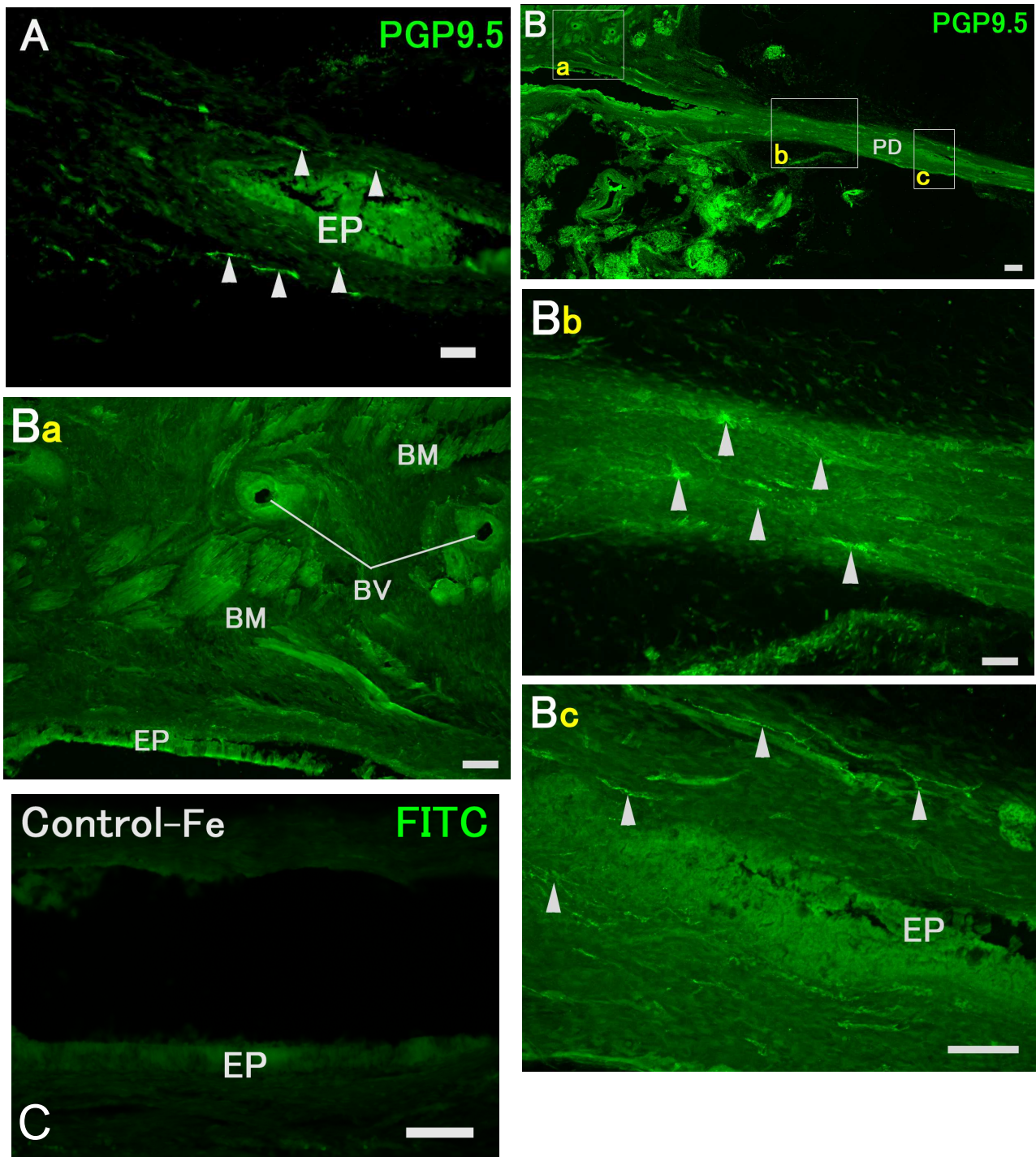
Fig. 1. PGP9.5 Expression in the human fetus parotid duct. **(A)** Magnified view of the parotid duct traversing over masseter in 5 month old fetus. **(B)** Whole image of 6 month old fetus parotid duct. Scale bar: 300 μm. **(Ba)** Magnified view of the corresponding box in (B) where the parotid duct penetrates buccinator muscle. Scale bar: 100 μm. **(Bb)** Magnified view of the corresponding box in (B) traversing over masseter. Scale bar: 100 μm. **(Bc)** Magnified view of the indicated area in (B) traversing over masseter closer to parotid gland. Scale bar: 100 μm. **(C)** Control (FITC) expression in the parotid duct traversing over masseter in 5 month old fetus. PD: parotid duct; BM: buccinator muscle; BV: blood vessels; EP: epithelium; Arrow heads: nerve fibers.

(Fig. 2A); however, the amount of nerve fibers was reduced where the duct penetrates the buccinator (Fig. 2B).

Expression of choline athetyltransferase (ChAT)

Since PGP9.5 antibody expression indicated that nerve fibers in adult parotid duct walls were mostly distributed immediately outside of the buccinator muscle, we investigated the type of nerve fibers by staining them with ChAT antibody. As a result, the majority of those nerve fibers reacted, which indicates that they are parasympathetic nerve fibers (Fig. 3A); the amount of ChAT reaction of the duct section penetrating the buccinator
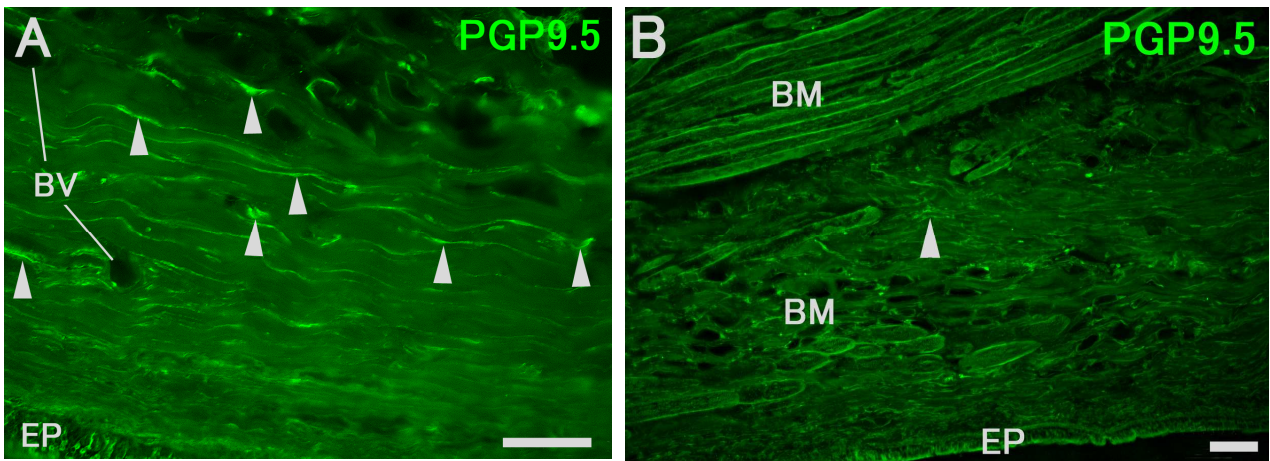
Fig. 2. PGP9.5 Expression in the human adult parotid duct. (**A**) Parotid duct section traversing over masseter. **(B)** Parotid duct section penetrating buccinator muscle. Scale bar: 100 μm. BM: buccinator muscle; BV: blood vessels; EP: epithelium; Arrow heads: nerve fibers.

Fig. 3. ChAT expression in the adult parotid duct. **(A)** Parotid duct section traversing over masseter. **(B)** Parotid duct section penetrating buccinator muscle. Scale bar: 100 μm. BM: buccinator muscle; 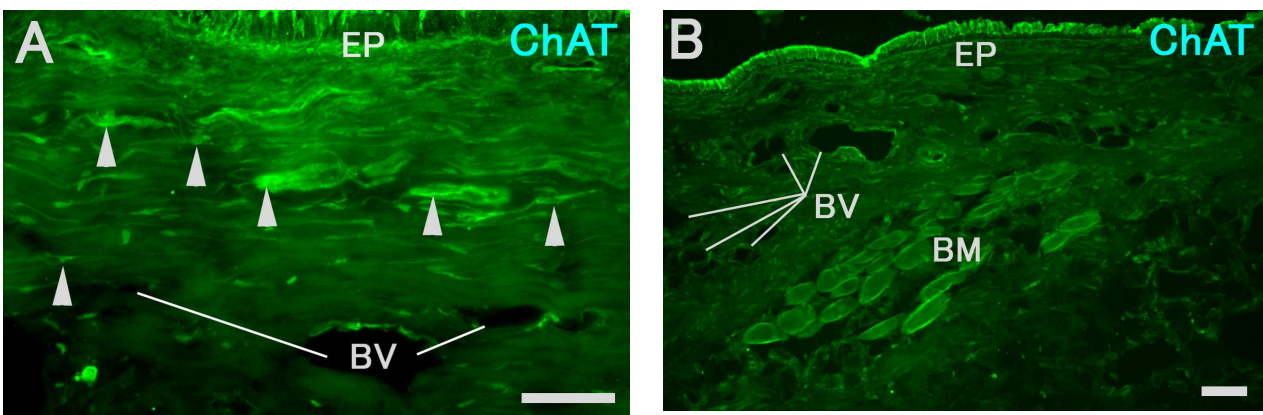BV: blood vessels; EP: epithelium; Arrow head: nerve fibers. **(C)** (Control) Control-Ad (FITC) expression in the human adult parotid duct traversing over masseter.
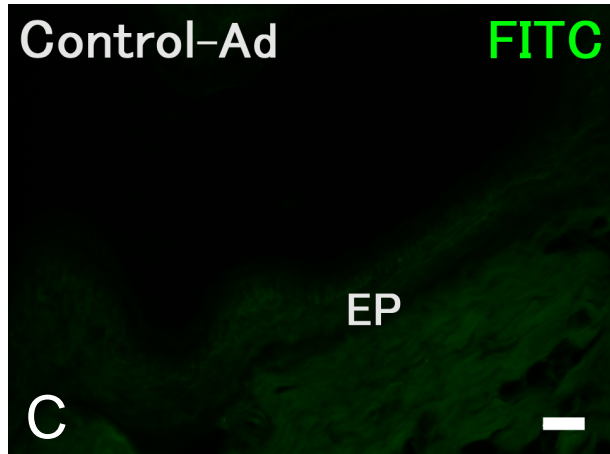
was lower, portraying much less concentration of parasympathetic nerve fibers in this area (Fig. 3B).

Expression of tyrosine hydroxylase (TH)

Some sympathetic nerve fibers reacted to TH antibody along blood vessels which are typically seen surrounding the parotid duct in the section of the parotid duct crossing over the masseter before entering the bucinator (Fig. 4A). Similar nerve fiber distribution pattern was observed with TH antibody staining as with PGP9.5 and ChAT in the area penetrating the buccinator (Fig. 4B). The section of the duct closer to the parotid gland consisted of more sympathetic nerve fibers reacting to TH antibody than any other area of the parotid duct (Figs. 4C, 4Ca).

DISCUSSION

Is the parotid duct merely a passageway for saliva after being secreted by the gland and its com-

position regulated by intraglandular duct? Only few detailed reports exist regarding the functions of the main duct; however, they all suggest that it has a special function other than providing a smooth passageway for saliva (Kutta et al., 2006; Kang et al., 2006; Amano et al., 2013). Salivary secretion is controlled by sympathetic and parasympathetic nerves of the autonomic nervous system. Parasympathetic innervation to the salivary glands is carried via the cranial nerves. The parotid gland receives its parasympathetic input from the glossopharyngeal nerve via the otic ganglion. The sympathetic nervous system also affects salivary gland
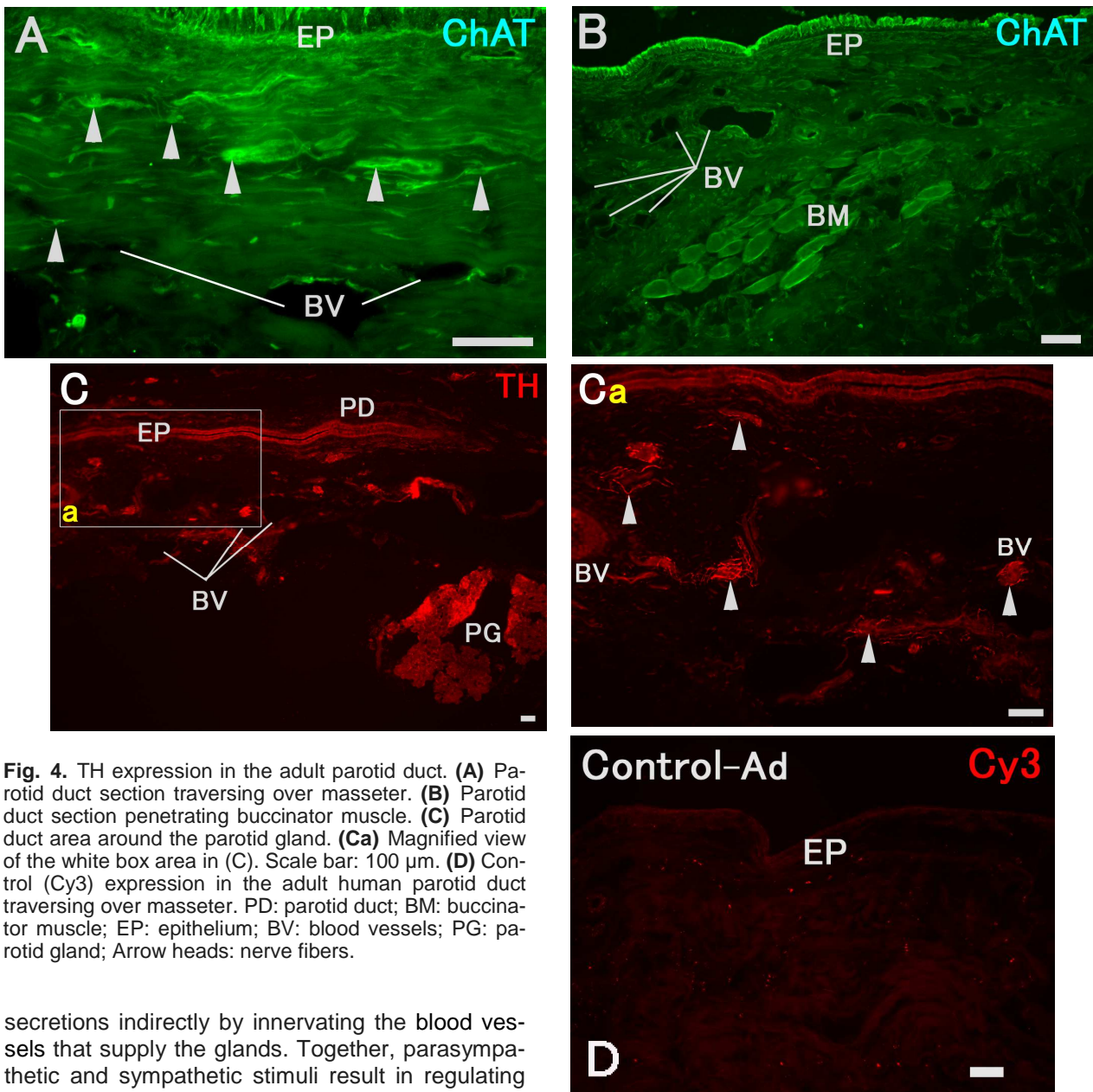
Fig. 4. TH expression in the adult parotid duct. **(A)** Parotid duct section traversing over masseter. **(B)** Parotid duct section penetrating buccinator muscle. **(C)** Parotid duct area around the parotid gland. **(Ca)** Magnified view of the white box area in (C). Scale bar: 100 μm. **(D)** Control (Cy3) expression in the adult human parotid duct traversing over masseter. PD: parotid duct; BM: buccinator muscle; EP: epithelium; BV: blood vessels; PG: parotid gland; Arrow heads: nerve fibers.

secretions indirectly by innervating the blood vessels that supply the glands. Together, parasympathetic and sympathetic stimuli result in regulating salivary gland secretions (Avery, 2001; Antonio, 2003; Junqueira and Carneiro, 2003; Ross et al., 2003). Once secreted by the gland, the composition of the saliva is modified prior to entering the intraglandular duct (Antonio, 2003; Proctor and Carpenter, 2007). Saliva is known to have strong antimicrobial effect and contains growth factors such as Epidermal Growth Factor (EGF) and Nerve Growth Factor (NGF), which assist in repairing wounds and enhancing regenerations of tissues (Dutta et al., 1992; Donnerer et al., 1992; Mathison et al., 1994; Woolf et al., 1994; Nagy, 2003; Jahovic et al., 2004; Oudhoff et al., 2008).

It has been reported that Trefoil Factor Family (TFF) peptides which are typically expressed in the intestinal mucosa such as stomach and colon are also expressed in the salivary gland (Madsen et al., 2007; Kouznetsova et al., 2010), and can contribute to protection of teeth and overall oral health (Storesund et al., 2009). Kutta et al. (2006) reported that the parotid duct has an antimicrobial defense mechanism, in which TFF peptides in the duct epithelium disturbs increased risk of lithogenesis. It is logical to suggest that if TFF peptides are found in intestinal mucosa to promote protection and repairs of the intestine, then mucous membrane of the parotid duct, another tubular structure of the digestive tract, should also contain similar protective proteins of the parotid duct. Furthermore, the roles and functions of the main ducts in the salivary glandular system are not just to provide a passage for saliva, but more importantly to regulate its flow.

Since the parotid duct had been perceived as a passageway for saliva, studies of its nerve distribution have been limited. Takeda conducted a rare study by histologically observed the parotid duct except for the area it penetrates the buccinator

muscle, and reported the presence of smooth muscle in the duct wall (Takeda, 1987). His results and our findings of nerve fibers abundant in the parotid duct except for the area penetrating the buccinator both support the notion that there is a correlation between smooth muscle and nerve distribution. Furthermore, Takeda suggests from the abundance of blood and lymph vessels surrounding the parotid duct that these vessels may act as an immunological barrier to the ascending infection of the parotid duct.

The nerve distribution of the human parotid gland has been observed by Heym and Matsuda using PGP9.5, TH and other antibodies (Heym et al., 1994; Matsuda et al., 1997), and TH expression throughout the parotid gland and its surrounding blood vessels has been reported (Heym et al., 1994). Our own observations of TH positive distribution in the areas close to the parotid gland and its surrounding blood vessels, i.e., abundance of sympathetic nerves in the blood vessel wall, indicate that there may be a correlation between sympathetic nerves and salivary flow regulation. We reported the lack of smooth muscle in the area of the parotid duct where it penetrates the buccinator muscle in our previous study (Amano et al., 2013). In this study, we observed a very small amount of nerve fibers in the same area in both fetus and adult specimens, leading us to speculate that there is a direct correlation between the amount of nerve fibers and smooth muscle in the section of the parotid duct where it penetrates the buccinator. Results from our previous study, the absence of smooth muscle fibers in the section of the adult parotid duct wall where it penetrates through the buccinator muscle, also supports our notion that the parotid duct acts as a sphincter to control the flow of saliva, manipulated by its surrounding buccinator muscle as it contracts and expands (Amano et al., 2013).

The sphincter mechanism of the duct is also supported by the results of this study, a sudden significant decrease in the number of nerve fibers in the parotid duct. Like the sphincter of Oddi, the buccinator, a skeletal muscle, surrounds a section of the duct which reacts to its movement similar to that during swallowing and mastication. In contrast, abundance of nerve fibers both in the duct section close to the gland and the section crossing over the masseter muscle indicate that those sections of the parotid duct have an aggressive role in the assistance of salivary passage. Furthermore, the heavy concentration of sympathetic nerve fibers along with blood vessels compared to other parts of the duct may imply that this particular section is more actively involved in the regulation of salivary flow.

From a developmental aspect, we suggest that the nerve distribution of the parotid duct has already begun by five months of gestation based on our data. Fetuses are known to begin movement as early as four to five months of gestation and suckling reflex, which is considered the first stage of oral function, around the sixth month (Sadler, 1995; Carlson, 2004). These movements likely lead to muscle growth as well as other structural development.

In conclusion, we investigated the nerve distribution in the human adult and fetal parotid ducts using PGP9.5, and used TH and ChAT in adult parotid ducts to further break down the distribution into sympathetic and parasympathetic nerve fibers. PGP9.5 staining revealed an abundance of nerve fibers throughout the entire duct; and ChAT and TH antibody staining identified a dense concentration of parasympathetic and sympathetic nerves except for the section where it penetrates the buccinator muscle. These results suggest that salivary secretion from the parotid gland and the regulation of salivary flow in the parotid duct are controlled by the autonomic nervous system, with the exception of this particular area penetrating the buccinator. From the significant decrease in the number of both sympathetic and parasympathetic nerve fibers in this area, we suggest that the duct function of this particular area is controlled by skeletal muscle, i.e., buccinator muscle movement. Further investigation of the types of nerves present in this area will assist us in understanding more clearly how the buccinator and this section of the parotid duct function together as a sphincter of salivary flow.

REFERENCES

AMANO K, MORIYAMA H, SHIMADA K (2010) Morphological study of the fetal parotid duct and buccinators muscle and the relationship to salivary secretion. *Clin Anat*, 23: 642-648.

AMANO K, MORIYAMA H, SHIMADA K, MATSUMURA G (2013) Study of human duct in the area penetration through buccinators muscle and their functional relationship as a sphincter. *Ital J Anat Embryol.* 118: 6-8.

ANTONIO N (2003) Ten Cate's Oral Histology. Development, structure, and function. Sixth Edition, pp 299-328.

AVERY JK (2001) Oral Development and Histology. Third Edition, pp 292-330.

CARLSON BM (2004) Human Embryology and Developmental Biology. Third Edition, pp 267-275, 483-484.

CARPENTER GH, PROCTOR GB, EBERSOLE LE, GARRETT JR (2004) Secretion of IgA by rat parotid and submandibular cells in response to autonomimetic stimulation in vitro. *.Int immunopharmacol.* 4: 1005-

1014.

DONNERER J, SCHULIGOI R, STEIN C (1992) Increased content and transport of substance P and calcitonin gene-related peptide in sensory nerves innervating inflamed tissue: evidence for a regulatory function of nerve growth factor in vivo. *Neuroscience*, 49: 693-698.

DORAN JF, JACKSON P, KYNOCH PA, THOMPSON RJ (1983) Isolation of PGP9.5, a new human neurone-specific protein detected by high-resolution two dimensional electrophoresis. *J Neurochem*, 40: 1542-1547.

DUTTA SK, ORESTES M, VENGULEKUR S, KWO P (1992) Ethanol and human saliva: effect of chronic alcoholism on flow rate, composition, and epidermal growth factor. *Am Coll of Gastroenterology*, 87: 350-354.

EKSTRÖ MJ (1989) Autonomoc control of salivary secretion. *Proc Finn Dent Soc*, 85: 323-331.

GARRETT JR, KIDD A (1993) The innervation of salivary glands as revealed by morphological methods. *Microsc Res Tech,* 26: 75-91.

HEYM C, WEBBER R, ADLER D(1994) Immunocytochemical correlation of peptides and tyrosine hydroxylase in nerve fibers of the human parotid gland. *Arch Oral Biol*, 39: 213-221.

JACKSON P, THOMPSON RJ (1981) The demonstration of new human brain specific protein by hifh-resolution two-dimensional polyacrylamide gel electrophoresis. *J Neurol Sci*, 49: 429-438.

JAHOVIC N, GÜZEL E, ARBAK S, YEĞEN BC (2004) The healing-promoting effect of saliva on skin burn is mediated by epidermal growth factor (EGF): Role of the neutrophils. *Burns*, 30: 531-538.

JUNQUEIRA LC, CARNEIRO J (2003) Basic Histology, Text and Atlas. Tenth Edition, pp 325-331.

KANG HC, KWAK HH, HU KS, YOUN KH, JIN GC, FONTAINE C, KIM HJ (2006) An anatomical study of the buccinator muscle fibers that extend to the terminal portion of the parotid duct, and their functional roles in salivary secretion. *J Anat*, 208: 601-607.

KONTTINEN YT, SORSAT, HUKKANEN M, SEGERBERG M, KUHLEFELT-SUNDSTÖM M, POLAK JM (1992) Topology of innervation of labial salivary glands by protein gene product 9.5 and synaptophysinimmunoreactive nerve in patients with Sjögren's syndrome. *J Rheumatol*, 19: 30-37.

KOUZNETSOVA I, GERLACH KL, ZAHL C, HOFFMANN W (2010) Expression analysis of human salivary glands by laser microdissection: Differences between submandibular and labial glands. *Cell Physiol Biochem*, 26: 375-382.

KUTTA H, MAY J, JAEHE M, MÜNSHER, PAULSEN P (2006) Antimicrobial defence mechanisms of the human parotid duct. *J Anat*, 208: 609-619.

LAURIA G, BORGNA M, MORBIN M, LOMBARDI R, MAZZOLENI G, SGHIRLANZONI A, PAREYSON D (2004) Tubule and neurofilament immunoreactivity in human hairy skin: markers for intra epidermal nerve fibers. *Muscle Nerve*, 30: 310-306.

MADSEN J, NIELSEN O, TORNÖEITHIM L, THIM L, HOLMSKOV U (2007) Tissue localization of human trefoil factors 1,2, and 3. *J Histochem Cytochem*, 55: 505-513.

MATHISON R, DAVISON J S, BEFUS A D (1994) Neuroendocrine regulation of inflammation and tissue repair by submandibular gland factos. *Immunol Today*, 15: 527-532.

MATSUDA H, KUSAKABE T, KAWAKAMI T, AGAHARA T, TAKENAKA T, TSUKUDA M (1997) Neuropeptide-containing nerve in the human parotid gland: a semi-quantitative analysis using an antibody against protein gene product 9.5. *Histochem J*, 29: 539-544.

NAGY G (2003) Role of saliva: Salivary glands and epidermal growth factor (EGF) on oral wound healing. *Fogorv Sz*, 96: 17-20.

OUDHOFF MJ, BOLSCHER JG, NAZMI K, KALAY H, VON'T HOF W, AMERONGEN AV, VEERMAN EC (2008) Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assy. *FASEB J*, 22: 3805-3812.

PROCTOR GB, CARPENTER GH (2007) Regulation of salivary gland function by autonomic nerves. *Autonomic Neuroscience: Basic and Clinical*, 133: 3-18.

PROTO AF Jr, PROUD GO, NORRIS CW, ODOI H (1981) Secretory innervation of the parotid gland. *Otolayngol Head Neck Surg,* 89: 16-19.

ROSS MH, KAYE GI, PAWLINA W (2003) Histology. A Text and Atlas. Fourth Edition, pp 455-461.

SADLER TW (1995) Langman's Medical Embryology. Seventh Edition, pp 90-99.

SHIMAMURA A (1957) Age determination and physical measurements of Japanese embryo. *Jpn J Legal Med*, 11: 795-811.

STORESUND T, SCHREURS O, MESSELT EB, KOLLTVEIT KM, SCHENCK K (2009) Trefoil factor family 3 expression in the oral cavity. *Eur J Oral Sci*, 117: 636-643.

TAKEDA Y (1987) Histoarchitecture of the human parotid duct. *Acta Anat*, 128: 291-294.

THOMPSON RJ, DORAN JF, JACKSON P, DHILLON AP, RODE J (1983) PGP9.5-a new marker for vertebrate neurons and neuroendocrine cells. *Brain Res*, 278: 224-228.

THULIN A (1976) Motor and secretory effects of nerves on the parotid gland of the rat. *Acta Physiol scand*, 96: 506-511.

TSUBOI T, HONDA T, HISHIDA S, SHIGETOMI T, UEDA M, SUGIURA Y (2004) A quantitative study of nerve fiber density in the submandibular gland of rat. *Nagoya J Med Sci*, 67: 25-34.

WANG L, HILLIGES M, JERNBERG T, WIEGLEB-EDSTRÖ D, JOHANSSON O (1990) Protein gene product 9.5-immunoreactive nerve fibers and cell in human skin. *Cell Tissue Res*, 261: 25-33.

WOOLF CJ, SAFIEH-GARABEDIAN B, MA Q-P, CRILLY P, WINTER J (1994) Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. *Neuroscience*, 62: 327-331.