Distribution of VIP receptors in the human submandibular gland: an immunohistochemical study

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Summary. Distribution of vasoactive intestinal polypeptide (VIP)-immunoreactive nerve fibers and VIP-receptor (VIP-R)-immunoreactive sites in the human submandibular gland were examined by the peroxidase-antiperoxidase method using the specimens taken from patients that had not received radiotherapy.

VIP-immunoreactive fibers were found around both serous and mucous acini, the duct system, and those around the mucous acini were more numerous than those around the serous acini. VIP-R immunoreactivity was restricted to the mucous acini and the intercalated duct segment. The serous acini, striated duct, and excretory ducts lacked VIP-R immunoreactivity. These findings suggest that the mucous acinar cells contain VIP-R, and that VIP-R-mediated VIP action is involved in regulating synthesis of viscous saliva and its release.

Key words: Submandibular gland, VIP, VIP receptor, Immunohistochemistry, Humans

Introduction

The human submandibular glands are under the peptidergic innervation. Vasoactive intestinal polypeptide (VIP)-, neuropeptide Y (NPY)-, galanin (GAL), calcitonin gene-related peptide (CGRP)-, and substance P (SP)-immunoreactive nerve fibers are distributed around the acini, ducts, and blood vessels, although there are some differences in the distribution and abundance (Lundberg et al., 1988; Hauser-Kronberger et al., 1992; Salo et al., 1993; Kusakabe et al., 1997). In the rat submandibular glands, VIP stimulates mucin release (Turner and Camden, 1990), and the protein-rich, viscous saliva secretion is enhanced by treatment with a combination of VIP and SP (Bobyock and Chernick, 1989; Ekström and Tobin, 1989). *In vitro*, tissues of rat submandibular glands released protein in response to

NPY (Ekström et al., 1996). In addition, VIP increased cyclic AMP production in the human submandibular gland (Larsson et al., 1986). Based on this, it has been considered that regulation of the synthesis of secretory products and their release in the human submandibular gland may be under the dense peptidergic innervation (Lundberg et al., 1988; Hauser-Kronberger et al., 1992; Salo et al., 1993; Kusakabe et al., 1997).

The human submandibular gland is a mixed gland which possesses many serous acini and a small number of mucous acini, but previous reports on this gland have described the distribution of neuropeptide-containing fibers without attention to the precise classification of the acinar structure. Our recent study of the human submandibular gland showed that VIP-, NPY-, and GAL-containing fibers were more numerous around the mucous acini than around the serous acini (Kusakabe et al., 1997). The difference in the distribution of peptidergic fibers between the mucous and serous acini may be reflected by the different distribution of the peptide-receptor sites in these acini. To clarify this, in the present study, the occurrence and distribution of VIP-receptors (VIP-R) were immunohistochemically examined in the human submandibular gland.

As we recently suggested (Kusakabe et al., 1997), it is necessary to state clearly the condition of the patients to obtain reliable results in the human tissue, because Forsgren et al. (1992) and Franzen et al. (1993) have suggested that irradiation causes fluctuation of neuropeptide immunoreactivity. For this reason, the present study was performed using specimens taken from patients that had not received radiotherapy.

Materials and methods

Tissue preparation

Specimens of human submandibular glands were obtained from a total of 5 patients (a 45-year-old male, a 52-year-old female, a 58-year-old female, a 64-year-old male, and a 71-year-old male) suffering from laryngeal or hypopharyngeal cancer. None of the patients had been

treated with radiotherapy before operation. The surgical tissue samples removed by radical neck dissection were immediately fixed by immersion in 4% para-formaldehyde and 0.2% picric acid in 0.1M phosphate buffer saline (PBS), pH 7.3, at 4 °C. Under a dissecting microscope, normal gland tissues were taken from these specimens, sliced into small blocks (5x5x2 mm), and immersed in the same fixative for 8 h. Dissection was carried out in cold fixative within a few minutes. After a brief washing with PBS, the specimens were transferred to 30% sucrose in PBS and kept there overnight at 4 °C. The specimens were then sectioned at 10 μm on a cryostat, and mounted on poly-L-lysine-coated slides. To confirm whether the conditions of fixation were good or not, and whether the obtained tissue samples had been invaded by cancer cells or not, some sections taken from all samples were stained with hematoxylin and eosin, and only normal samples were processed for immuno-histochemistry. We have previously confirmed that this protocol is sufficient to obtain well-fixed human tissues for reliable immunohistochemistry (Kusakabe et al., 1997).

For observation of VIP-R immunoreactivity, serial sections were mounted on two series of slides. One was stained with VIP-R antiserum and the other with hematoxylin and eosin.

Immunohistochemistry

The sections were processed for immunohisto-chemistry according to the peroxidase-antiperoxidase (PAP) method. Prior to PAP treatment, sections were dipped in a fresh 0.3% solution of hydrogen peroxide in methanol for 30 min at room temperature to inhibit endogenous peroxidase activity. After washing in several changes of 0.3% Triton-X in 0.1M PBS (PBST), the sections were treated for 1 h with a protein blocking agent (Immunoson, USA) at room temperature to block nonspecific protein binding sites. Then they were incubated at 4 °C overnight with rabbit polyclonal antiserum against porcine VIP (Incstar, USA) and rat monoclonal antiserum against VIP-R (Immunotech, USA) derived from human adenocarcinoma cells. The antisera were diluted to 1:1500 (VIP) and 1:50 (VIP-R) with 0.2% bovine serum albumin, 1% normal goat serum, and 0.2% sodium azide in PBST. After rinsing in several changes of PBST, the sections were transferred for 2 h to anti-rabbit IgG or to anti-rat IgG diluted to 1:200 with 0.2% bovine serum albumin, 1% normal goat serum, and 0.2% sodium azide in PBST at room temperature. Next, the sections were rinsed with several changes of PBS, transferred for 2 h to rabbit PAP complex (Jackson, USA) or to rat PAP complex (Jackson, USA) diluted to 1:200 with 0.2% bovine serum albumin, 1% normal goat serum, and 0.2% thimerosal in PBS, and rinsed in several changes of PBS. The peroxidase activity was demonstrated with 3,3'-diaminobenzidine. This immunostaining procedure has been detailed in a previous report (Kusakabe et al.,

1991).

To clearly discriminate the serous and mucous acini, alternate immunostained sections were briefly counter-stained with hematoxylin and eosin.

Control

VIP antiserum was preincubated with 50 μm of the synthetic VIP (Sigma, USA). The absorbed antiserum was used for incubation of the sections followed by incubation with the secondary antiserum to test the specificity of the primary antiserum. Sections were incubated with normal rat serum instead of the monoclonal VIP-R antiserum.

Results

Immunoreactivity of VIP was seen in the nerve fibers mainly distributed around the secretory components, the serous and mucous acini, and duct systems of the human submandibular gland. In briefly counterstained sections, VIP-immunoreactive nerve fibers around the mucous acini were more numerous than around the serous acini (Fig. 1) as we have recently reported (Kusakabe et al., 1997).

In low magnification views of two serial sections, one stained with hematoxylin-eosin (Fig. 2A) and the other immunostained for VIP-R (Fig. 3A), VIP-R immunoreactivity was recognized in some clusters of acinar cells (Fig. 3A). Comparing these serial sections at high magnification, the location of the VIP-R immuno-reactive cell clusters coincided with that of the mucous acinar regions (Figs. 2B, 3B), because the mucous acinar cells with serous demilunes were easily discriminated in the sections stained with hematoxylin-eosin (Fig. 2B). The mucous acinar cells demonstrated intense VIP-R immunolabelling of the luminal and basal membranes (Figs. 3B, 4). The serous acinar cells lacked VIP-R immunoreactivity (Fig. 3B), although the short segment of intercalated ducts which were connected with the serous acini showed intense VIP-R immunoreactivity (Fig. 3B,C). The striated, intralobular, and interlobular excretory ducts also lacked VIP-R immunoreactivity (Fig. 3A,C). Inter- and intralobular connective tissue, and periductal myoepithelial cells displayed only faint staining at background level. These views were supplementary by briefly counterstained sections (Fig. 4).

Discussion

The present study clearly showed intense immuno-reactivity of VIP-R in the mucous acini and intercalated duct segment of the human submandibular gland. Herbst et al. (1992) reported that VIP-R immunoreactivity is detected in the mucous acini of the human palatal and labial glands, which are mixed salivary glands, and in the intercalated duct segment of the human parotid gland. The present immunostaining of VIP-R is in good

accord with their findings. Thus, the presence of VIP-R immunoreactivity in the human mixed glands may be restricted to the mucous acini. The secretory function of the submandibular gland is under the control of the autonomic nervous system. The sympathetic nerve stimulation causes the secretion of a small quantity of viscous saliva, but stimulation of the parasympathetic nerve, on the contrary, yields abundant serous saliva (Pinkstaff, 1980). Considering this, together with the present findings, it makes us suppose that the sympathetic nerves innervating the mucous acini may contain VIP, and that the parasympathetic cholinergic nerve may not contain VIP. However, the adrenergic nerve fibers probably do not contain VIP, because, as far as we are aware, there are no immuno-histochemical reports showing the coexistence of tyrosine hydroxylase and VIP in the sympathetic nerves. Accordingly, it seems likely that nonadrenergic VIP-containing nerve fibers terminate in the mucous acini with VIP-R. Thus, the regulating synthesis of viscous saliva and its release (Bobyock and Chernick, 1989; Ekström and Tobin, 1989; Turner and Camden, 1990) may be caused by VIP-R- mediated VIP action, and may be modulated by a cyclic AMP second messenger system, as suggested in

the human submandibular gland (Larsson et al., 1986) and in the rodent exocrine pancreas (Deschodt-Lanckman et al., 1975; Pearson et al., 1981).

On the other hand, the physiological significance of VIP-R in the intercalated duct is unclear, because the function of the intercalated duct segment in the exocrine gland is also unclear. Herbst et al. (1992) showed VIP-R immunoreactivity in some excretory duct cells in addition to the intercalated duct cells, but the intralobular and interlobular excretory duct cells of the human submandibular gland did not show VIP-R immunoreactivity. This disagreement may be due to the histological difference in these two glands; the human parotid gland is a purely serous gland whereas the submandibular gland is a mixed gland.

More recently, we have shown a significant difference in the distribution of VIP-, NPY-, and GAL-immunoreactive fibers between the mucous and serous acini of this gland (Kusakabe et al., 1997). The mean length of VIP, NPY, and GAL fibers around mucous acini was greater than that of these fibers around serous acini. On this basis, we speculated that the difference in abundance of peptidergic fibers between the mucous and serous acini may be reflected in the distribution of
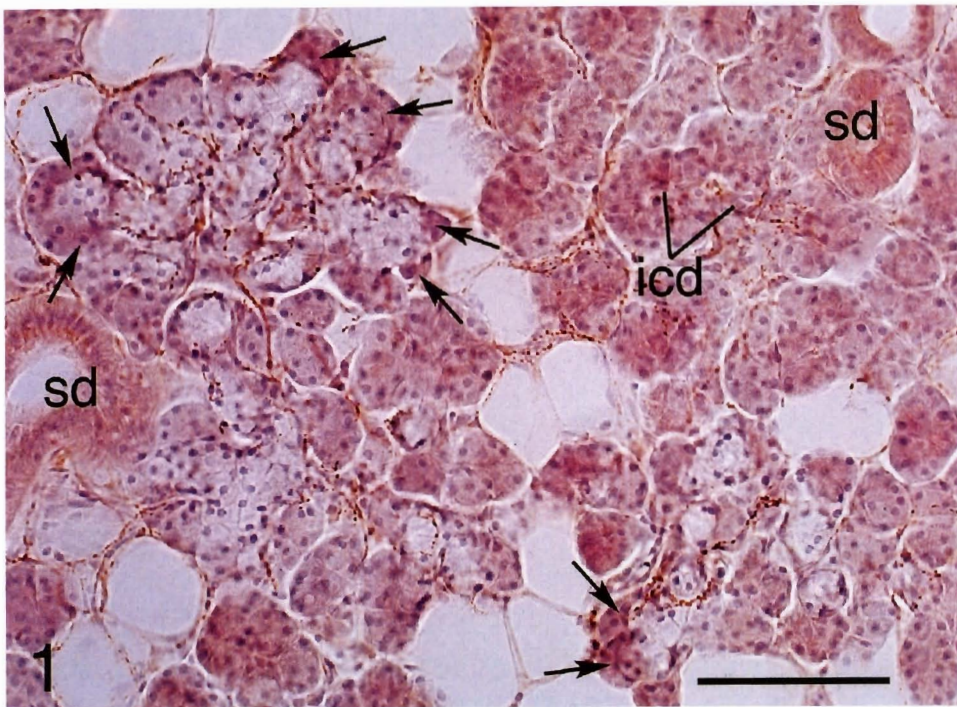

Fig. 1. A cryostat section of the submandibular gland briefly counter-stained with hematoxylin and eosin after immunostaining for VIP. The number of immunoreactive fibers around the mucous acini is higher than the number of those around the serous acini. Arrows indicate the serous demilunes. Scale bar: 100 μm.

Figs. 2 and 3. Two serial cryostat sections of the human submandibular gland stained with hematoxylin-eosin **(Fig. 2)** or VIP-R antiserum **(Fig. 3)**. 2B. Enlargement of area in 2A. Arrowheads indicate the serous demilunes. 3B and 3C. Enlargement of area in 3A. Two asterisk showing areas of mucous acini in 2B correspond to those in 3B. Mucous acinar cells show intense VIP-R immunoreactivity. Arrows in 3B and 3C indicate the short segment of the intercalated duct. Scale bar: 2A and 3A, 300μm; 2B, 3B and 3C, 100 μm.

Fig. 4. High magnification image of the human submandibular gland briefly counterstained with hematoxylin and eosin after immunostaining for VIP-R. Arrowheads indicate the serous demilunes. sd: striated duct. Scale bar: 220 μm.
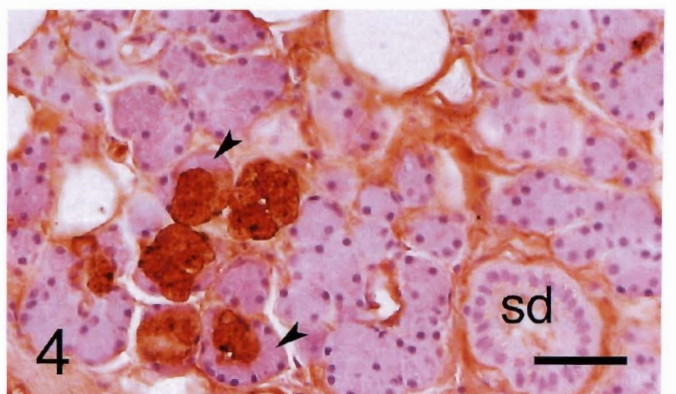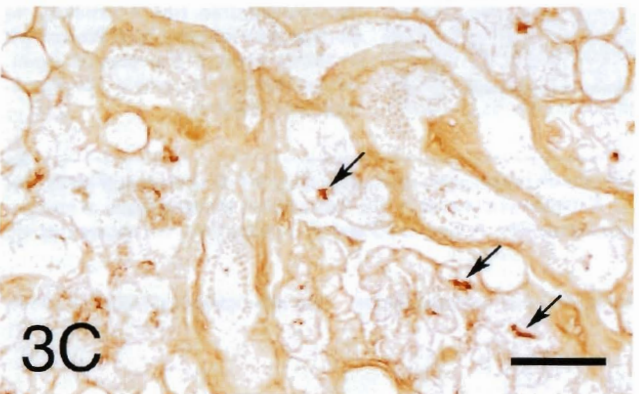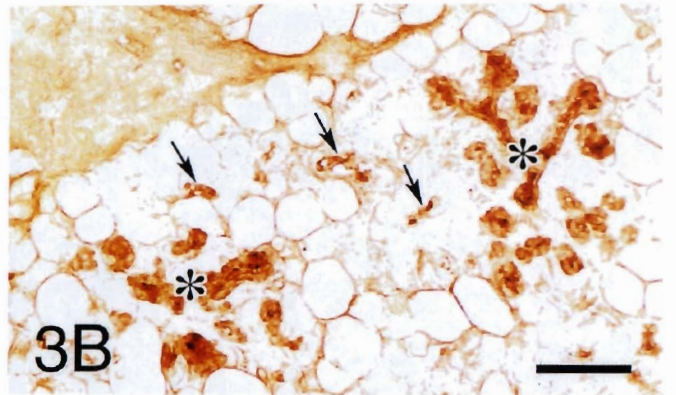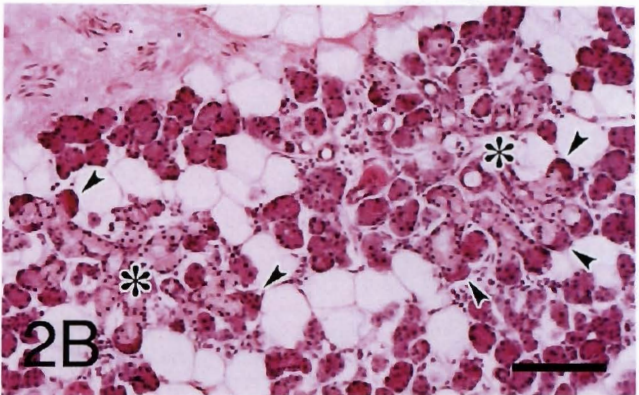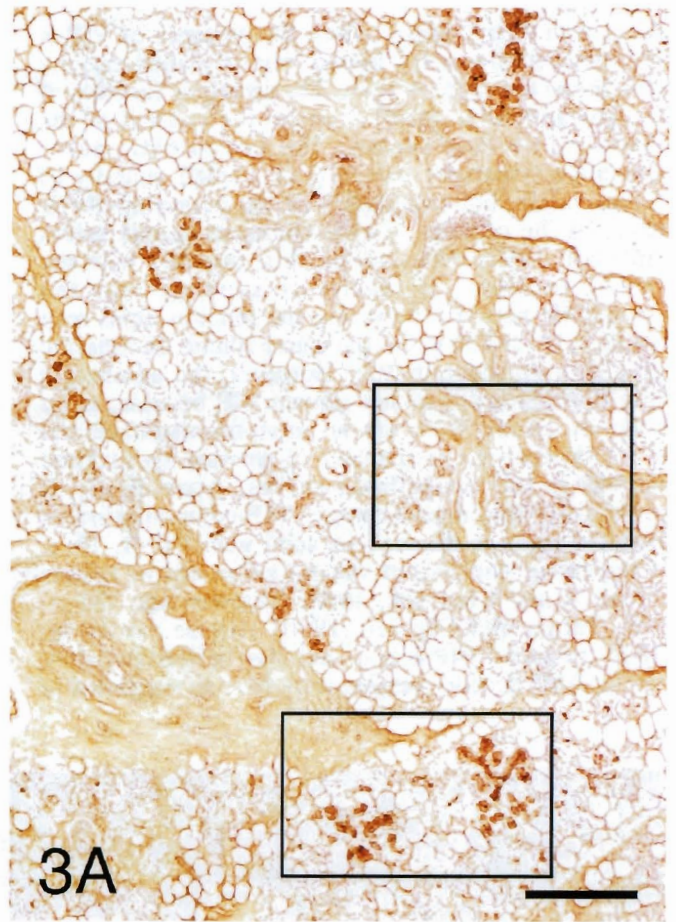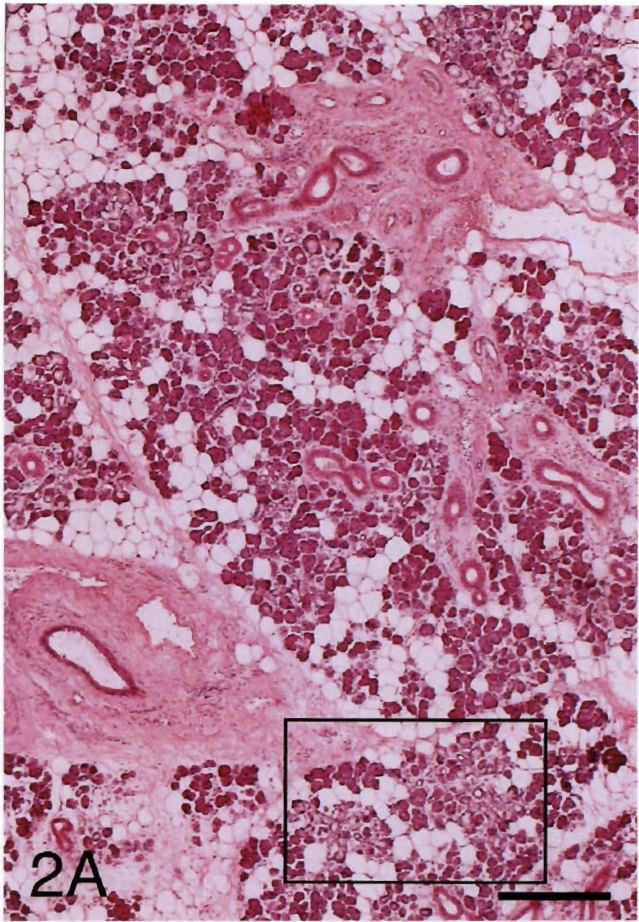
peptide-receptor sites in the mixed gland, as stated by Herbst et al. (1992). As far as the mucous acini are concerned, the present findings are in good agreement with the distribution of VIP fibers around the mucous acini. At least, this indicates that VIP-R-mediated VIP action is involved in regulating production of viscous saliva and its release as stated above. In the present study, however, VIP-R immunoreactivity was not detected in the serous acini. This indicates that the target of VIP-containing fibers in the human submandibular gland may be restricted to the mucous acinar cells and intercalated duct cells. However, previous studies on the human submandibular gland showed that VIP immuno-reactive fibers are present around the serous acini and the striated and excretory ducts as well as the mucous acini and intercalated duct (Lundberg et al., 1988; Hauser-Kronberger et al., 1992; Salo et al., 1993; Kusakabe et al., 1997). Thus, the lack of VIP-R immunoreactivity in the serous acini and striated and excretory ducts contracts with the distribution of VIP fibers around them. This contradiction may be explained by two possibilities: (1) VIP-fibers encircling the serous acini and striated and excretory ducts may be fibers on the way to the mucous acini or the intercalated duct cells; or (2) two different types of VIP-R may be present in the mucous and serous acini, respectively.

The first possibility can be clarified by a re-construction of a single VIP immunoreactive fiber using a confocal scanning microscope. On the other hand, with regard to the second possibility, two types of VIP-R (VIP-1 and VIP-2 receptor) have been recently cloned and sequenced (Usdin et al., 1994). The messenger RNA (mRNA) for VIP-1 and VIP-2 receptors has a different distribution, as mapped by *in situ* hybridization. VIP-1 receptor mRNA is detected in the lung, liver, small intestine, and brain (Ishihara et al., 1992), and VIP-2 receptor mRNA is predominantly found in the areas with neuroendocrine function (Lutz et al., 1993). Although we do not know whether the VIP-R monoclonal antiserum used in this study represents the chemical nature of VIP-1-R or VIP-2-R, the idea that immunologically different types of VIP-R molecules are present in the mucous and serous acini of the human submandibular gland may be helpful to solve this contradiction. Further molecular biological studies will make this clear.

In conclusion, the present results suggest that at least the mucous acinar cells contain VIP-R and can, in principle, bind VIP released from VIP-containing fibers around the mucous acini.

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