Localization of Connexin 26, 32 and 43 in Rat Parotid Glands

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Received for publication March 10, 1998

We investigated the distribution and localization of connexin 26, 32, and 43 in rat parotid glands. Immunofluorescence microscopy revealed the presence of spots reacting for connexin 26 and 32 between endpiece cells. A few spots indicating connexin 43 occasionally were observed in the parotid glands. Connexin 43-positive spots were localized around intercalated ducts. No positive spots for these connexins were detected between ductal cells. Double immunofluorescence microscopy revealed

that connexin 26-reactive sites could be observed within the same spots as connexin 32. Double immuno-electron microscopy confirmed that connexin 26 and 32 were co-localized in the same gap junction. These results suggest that connexin 26 and 32 are associated with secretory function and permeability between endpiece cells and that connexin 43 is associated with contraction of the myoepithelial cells that surround intercalated ducts in the rat parotid glands.

Key words: Connexin, Gap junction, Parotid gland, Acinar cell, Myoepithelial cell

I. Introduction

Gap junctions (GJs) are transmembrane sites that directly link adjacent cells and that mediate the intercellular movement of low molecular weight substances such as cAMP and Ca²⁺. Gap junctional intercellular communication is thought to play an important role in maintenance of homeostasis and coordination of secretion in multicellular organs [15]. GJs often are abundant in exocrine glands. It has been ultrastructurally confirmed that GJs are present between secretory cells in rat parotid (PGs) [6, 8], submandibular [5], and sublingual glands [18, 21, 22] as well as in bat PGs [24].

GJs are composed of connexon, which consist of protein assemblies referred to connexins (CXs). Many different CXs with varying degrees of sequence homology have been found. They are distinguished by reference to the CX molecular weight predicted from the corresponding cDNA [2, 3]. Recently, the distribution and localization of these CXs have been investigated in various organs by use of specific antibodies [4, 9, 12, 14, 16, 23, 26]. GJs between endpiece cells in exocrine glands consist of a 21 kDa protein called connexin26 (CX26) [26] and a 27 kDa protein called connexin32 (CX32) [9, 12, 20], while

those in endocrine glands are composed of a 43 kDa protein called connexin43 (CX43) [2, 3]. However, there have been only a few reports of CXs expression in rat salivary glands [10, 13, 16, 17]. Muramatsu *et al.* (1996) showed that CX32 was localized in GJs between endpiece cells and that CX43 was found between myoepithelial cells of rat submandibular and sublingual glands [17]. Hirono *et al.* (1995) demonstrated immunohistochemically the expression of CX26 and 32 between endpiece cells in rat PGs and that of CX26 in the intercalated duct [10]. However, this localization was not fully clarified at the immunocytochemical level. It is still an open question as to whether CX26 and 32 co-exist in the same GJ. Further, no report has documented the existence of CX43 in PGs, although myoepithelial cells are present on the intercalated duct in PGs [7].

The purpose of this study was to investigate the distribution and localization of CX26, 32 and 43 in rat PGs by means of immunohistochemistry and immunocytochemistry.

II. Materials and Methods

Animals and tissue preparation

PGs were obtained from 32 adult male Sprague-Dawley rats weighing about 300 g that were under deep anesthesia induced by sodium thiopental. After dissection, PGs were quickly frozen in liquid nitrogen and stored

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at -80°C for immunohistochemical experiments. The immunocytochemical procedures were carried out according to Koike *et al.* (1993) [11]. Perfusion fixation with 1% paraformaldehyde-0.1 M phosphate buffer (PB) was performed transcardially. The PGs samples were cut into small pieces and left in the same fixation for 5 hr.

Antibodies

Polyclonal antibody anti-CX26 (amino acid sequence 101–119) was kindly supplied by Prof. Y. Shibata and Dr. A. Kuraoka (Kyushu University, Fukuoka, Japan). The characteristics and specificity of this antibody have previously been demonstrated [14]. Mouse monoclonal antibody anti-CX32 (6–3G11) was supplied by the courtesy of Dr. F. Ueda (Nippon Shinyaku Co., Kyoto, Japan). This antibody binds to a cytoplasmic epitope, the carboxyl-terminal domain of CX32 [23]. Mouse monoclonal antibody anti-CX43 (amino acid sequence 252–270, Zymed Laboratories, San Francisco, CA) was used.

Immunohistochemistry

For immunohistochemistry, indirect immunofluorescence was performed on non-fixed cryosections according to the method of Muramatsu *et al.* (1996) [17] with modification. In brief, PGs were embedded into O.C.T. compound (Tissue Tek; Miles, Elkhart, IN) and quickly frozen in liquid nitrogen. They were sectioned at $6\text{ }\mu\text{m}$ at -20°C , and were mounted on silane-coated glass slide. Non-specific binding was blocked with 10% goat or rabbit serum in 0.01 M phosphate buffer saline (PBS) for 30 min, and then the specimens were incubated with anti-CX26 (diluted 1 : 100 in PBS), anti-CX32 (diluted 1 : 1000 in PBS), or anti-CX43 (diluted 1 : 1000 in PBS) antibodies for 12 hr at 4°C . After rinsing in PBS for 15 min, specimens were incubated with fluorescein isothiocyanate (FITC)-conjugated antibody (Cappel; Durham, NC) to mouse or rabbit IgG diluted 1 : 200 in PBS for 12 hr at 4°C . After washing, they were observed under a fluorescence microscope (Olympus BHT-RFK, Olympus, Tokyo, Japan).

Double immunofluorescent microscopy using both anti-CX32 and anti-CX26 antibodies was performed according to Kuraoka *et al.* (1993). After immunofluorescent staining with anti-CX32 and FITC-conjugated antibody as above, the same specimens were blocked with 10% goat serum in PBS for 30 min and incubated with anti-CX26 antibody for 24 hr at 4°C . After rinsing in PBS, specimens were incubated with rhodamine-conjugated goat anti-rabbit IgG (Tago; Burlingame, CA) diluted 1 : 50 in PBS for 24 hr at 4°C and observed under a fluorescence microscope.

For quantitation, color slides at a magnification of $\times 100$ were projected onto a photographic screen. The number of fluorescent spots was scored. Data were expressed as the number of immunolabeled spots per $10,000\text{ }\mu\text{m}^2$ of tissue. The data comparing CX26 with 32, CX26 with 43, and CX32 with 43 were analyzed statistically by

Mann-Whitney's U test. The criterion for statistical significance was $p < 0.01$.

Immunocytochemistry

For investigation of CX26 and 32 co-localization, the ultrathin cryosection method was utilized. Specimens were quickly frozen in liquid nitrogen after cryoprotection in 2.3 M sucrose-0.1 M PB for one day at 4°C , then cut into 80 nm-thick sections on a Reichert Ultracut-S equipped with an FC4E cryoattachment (Reichert, Vienna, Austria) at -100°C . The sections were mounted on 200 mesh nickel grids and rinsed in PBS for 2 hr and then blocked for 30 min with PBS containing 0.1 M glycine and 1% bovine serum albumin (PGB). They were incubated with anti-CX32 antibody for 12 hr at 4°C , washed in PBS for 2 hr at room temperature, and then incubated for 12 hr at 4°C with colloidal gold conjugated goat anti-mouse IgG (particle size : 5 nm, Zymed) diluted 1 : 20 in PGB. After rinsing in PBS for 2 hr, they were incubated with anti-CX26 antibody for 12 hr at 4°C . After washing in PBS in 2 hr, they were incubated with colloidal gold conjugated goat anti-rabbit IgG (particle size : 10 nm, Zymed) diluted 1 : 20 in PGB. The sections were post fixed in 1% OsO_4 -0.1 M PB for 1 hr after washing in PBS. They were dehydrated, embedded in acrylic resin (LR White, The London Resin, Hampshire, NW, UK), and after staining with uranyl acetate and lead citrate examined under a transmission electron microscope [JEOL 100CX, JEOL; Tokyo, Japan].

III. Results

Immunohistochemistry

A large number of fluorescent spots which had reacted positively for anti-CX26 and CX32 antibody were observed in endpieces in the rat PGs (Figs. 1, 2). In contrast, only a few CX43 spots were recognizable (Fig. 3). At higher magnification, punctate decoration of CX26 and 32 was detected within the endpieces, consistent with the lateral borders of the endpiece secretory cells (Figs. 1, 2, 4). Immunolabeling was absent from the luminal and basal borders of the endpiece cells. However, CX43-spots were observed around intercalated ducts (Fig. 5). No positive spots for any of the three types CXs were found between ductal cells (Figs. 1, 4, 5). Numerous green CX32 spots stained by FITC indicating CX32 were seen in the endpieces (Fig. 6). Red CX26 spots stained red by rhodamine were observed in the same glandular components (Fig. 7). Reactive spots for both CX26 and CX32 seemed to overlap as indicated by arrows in Figs. 6 and 7.

The mean number of immunofluorescent spots per $10,000\text{ }\mu\text{m}^2$ for CX26, CX32 and CX43 are graphically portrayed in Fig. 8. The means for CX26 and CX32 are not significantly different, but both differ significantly from that of CX43 ($p < 0.01$). This analysis confirms the subjective impression gained from Figs. 1–7 that the CX26- and CX32-positive spots are common, while those

Figs. 1–5. Fluorescent micrographs. Fluorescent spots indicating positive reaction with anti-CX26 (Fig. 1) and 32 (Fig. 2) antibodies are observed in endpieces of rat PGs. In contrast, CX43-reactive spots are rare, and their localization is unclear at low magnification (Fig. 3). At higher magnification, punctate decoration of CX26 and 32 is seen mostly within acini, consistent with the apposed lateral plasma borders of the endpiece secretory cells (CX32; Fig. 4, CX26; data not shown). A few CX43-reactive sites can be detected around an intercalated duct (Fig. 5). No positive spots for any connexin are detected between ductal cells in PGs (Figs. 1, 4, 5). Original magnifications: Figs. 1, 2, 3 $\times 280$; Fig. 4 $\times 350$; Fig. 5 $\times 420$, D: duct.

Figs. 6, 7. Fluorescent micrographs. Double-labeling immunofluorescent microscopy reveals that CX32-reactive sites (Fig. 6, FITC; green) correspond to the same sites where CX26 reacted (Fig. 7, rhodamine; red) in rat PGs (arrowheads show the same sites). No positive spots for both CXs are detected between ductal cells. Original magnifications: Figs. 6, 7 $\times 325$; D: duct.

of CX43 are rare.

Immunocytochemistry

In ultrathin cryosections, intracytoplasmic structures were poorly preserved, because the concentration of the fixative used in this study was lower than usual. However, the plasma membrane was relatively well-preserved. By using the presence of electron-lucent spaces indicating secretory granules or elongated endoplasmic reticulum as markers, it was possible to distinguish endpiece secretory cells from myoepithelial cells in the rat PGs. Immuno-

labeled particles for CX26 and 32 were observed at GJs between secretory cells of PGs in ultrathin cryosections. In double immuno-electron micrographs, 5 nm particles indicating CX32 and 10 nm gold particles revealing CX26 were found on or contiguous to GJs between endpiece cells (Fig. 9). The number of 5 nm gold particles was relatively greater than that of 10 nm particles. No orderly distribution of both particles was apparent.

IV. Discussion

There have been many morphological investigations on GJs in the salivary glands [18, 21, 22, 25]. GJs exist between acinar cells, between myoepithelial cells, and also

Fig. 8. The mean numbers of positive spots for CXs per 10,000 μm^2 in PG. CX26-positive spots are most common; in contrast, CX43-reactive spots are rare. There are statistically significant differences between CX26 and 43, and between CX32 and 43 by Mann-Whitney's U-test ($p < 0.01$).

Fig. 9. Electron micrograph. Double immuno-electron microscopy confirming that connexin 32 and 26 are co-localized in the same GJs between secretory cells of PGs in ultrathin cryosections. The gold particles are located at closely apposed plasma membranes of endpiece cells. Magnifications: $\times 300,000$; Bar = 0.1 μm , GJ, gap junction; Small (5 nm) gold particles show CX32-reaction; Large (10 nm) ones are CX26 localization.

between acinar cells and myoepithelial cells, but not between ductal cells [21]. On the other hand, some investigators have suggested the presence of GJs in ductal regions [25]. Recently, immunohistochemical analysis has become available for researching localization and distribution of GJ proteins in salivary glands. Hirono *et al.* (1995) reported that 21 kDa-GJ protein was localized at intercalated ducts in rat PGs and submandibular glands, but not observed in sublingual glands [10]. Our immunohistochemical results contradict these earlier studies in that we found no positive spots for any CX between ductal cells. Our finding is supported by the results of conventional electron microscopic and freeze-fracturing studies by Nagato and Tandler (1986) [18] and Shimono *et al.* (1980) [21] who found no GJs between ductal cells.

There are a few previous immunohistochemical studies of CX expression in the salivary glands, that showed CX26, CX32 and CX43 are present in the rat salivary glands [10, 13, 16, 17]. We previously demonstrated CX localization in the rat submandibular and sublingual glands [17]. The localization, distribution, and frequency of CX32 in submandibular and sublingual glands did not differ significantly. Kuraoka *et al.* (1994) indicated that CX26 and 32 were co-localized by double immunofluorescence in the same GJs in rat PGs; their data agree with our results [13]. However, our observations at the optical microscopic level might allow for varying interpretations. Therefore, we investigated the detailed localization of CX26 and 32 using immuno-electron microscopy to confirm whether both CXs were present in the same GJs. We were able to demonstrate the co-localization of both CXs in the same GJ between endpiece cells. These findings suggest that both CX26 and 32 are closely associated with secretory function in rat PGs. It is still not completely clear why CX26 and 32 are present between endpiece cells. One possibility is that the co-existence of both CX26 and 32 may be associated with phosphorylation. It has been surmised that CX32 has several phosphorylation sites, whereas CX26 is devoid of them. Further, Barrio *et al.* (1991) reported that CX26 exhibited different properties compared to CX32, and that the combination of CX26 and 32 resulted in differential control of the permeability of GJs [1]. Therefore, it is possible that phosphorylation of these CXs plays an important role in GJ permeability.

Our results also have shown that immunolabeling for CX26 and 32 was strong between secretory cells and that CX43 was very rare in rat PGs. Muramatsu *et al.* (1996) have demonstrated immunocytochemically that CX43 is abundant between GJs of myoepithelial cells in rat sublingual glands [17]. Comparing the results in this study with those in our previous study, the frequency of CX43 in PGs was definitely lower than that in both submandibular and sublingual glands. It is well established that myoepithelial cells invest only the intercalated ducts in the mature rat PG, although their processes often extend onto adjacent portions of acini and striated ducts [7, 19]. Thus, the observed localization of CX43 around the intercalated

ducts but not acini in rat PG suggests that CX43 marking GJs between myoepithelial cells processes and between these processes and intercalated duct cells.

V. Acknowledgments

The authors wish to thank Prof. Y. Shibata and Dr. A. Kuraoka for supplying CX26 antibody, Dr. F. Ueda for supplying CX32 antibody, Dr. Y. Enokiya and Dr. S. Hashimoto for technical assistance in electron microscopy, and Dr. T. Inoue for valuable criticism and discussions on this manuscript. This study was supported, in part, by Oral Health Science Center grant 961B03 from Tokyo Dental College.

VI. References

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