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Immunoelectron Microscopic Study of Podoplanin Localization in Mouse Salivary Gland Myoepithelium

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We have recently reported that salivary gland cells express the lymphatic endothelial cell marker podoplanin. The present study was aimed to immunohistochemically investigate the expression of the myoepithelial cell marker α-smooth muscle actin (SMA) on podoplanin-positive cells in mouse parotid and sublingual glands, and to elucidate podoplanin localization in salivary gland myoepithelial cells by immunoelectron microscopic study. The distribution of myoepithelial cells expressing podoplanin and α-SMA was examined by immunofluorescent staining, and the localization of reaction products of anti-podoplanin antibody was investigated by pre-embedded immunoelectron microscopic method. In immunohistochemistry, the surfaces of both the mucous acini terminal portion and ducts were covered by a number of extensive myoepithelial cellular processes expressing podoplanin, and the immunostaining level with anti-podoplanin antibody to myoepithelial cells completely coincided with the immunostaining level with anti-α-SMA antibody. These findings suggest that podoplanin is a salivary gland myoepithelial cell antigen, and that the detection level directly reflects the myoepithelial cell distribution. In immunoelectron microscopic study, a number of reaction products with anti-podoplanin antibody were found at the Golgi apparatus binding to the endoplasmic reticulum in the cytoplasm of myoepithelial cells between sublingual gland acinar cells, and were also found at the myoepithelial cell membrane. These findings suggest that salivary gland myoepithelial cells constantly produce podoplanin and glycosylate at the Golgi apparatus, and transport them to the cell membrane. Podoplanin may be involved in maintaining the homeostasis of myoepithelial cells through its characteristic as a mucin-type transmembrane glycoprotein.

Key words: podoplanin, salivary gland, myoepithelium, α-smooth muscle actin

I. Introduction

Lymphatic endothelial cell marker podoplanin was first identified as a 43-kd transmembrane protein of the kidney glomerular epithelial cells, the podocytes, and is homologous to the T1α-2 gene which encodes the type I alveolar cell specific antigen and to the oncofetal antigen M2A recognized by the D2-40 antibody [3, 4, 12, 20, 25, 26]. Podoplanin is first expressed at around E11.0 in Prox1-positive lymphatic progenitor cells [5, 9, 22]. Podoplanin (–/–) mice die at birth because of respiratory failure and have defects in lymphatic formation with diminished lymphatic transport and congenital lymphedema, but do not have defects in blood vessel pattern formation [28]. Podoplanin is a mucin-type glycoprotein negatively charged by extensive O-glycosylation and a high content of sialic acid [7, 10, 29–31]. The role of podoplanin has been studied in a rat model of nephropathy by puromycin aminonucleoside nephrosis with severe proteinuria. In the kidney with nephropathy, podocyte foot processes are extensive flat-

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tened and podoplanin is selectively reduced to <30%. It has been thought that podoplanin plays a role in maintaining the shape of podocyte foot processes and glomerular permeability because of the morphological alterations of cell shape with selective loss of podoplanin in nephrosis accompanies proteinuria [3, 11, 16, 17].

There have also been reports on podoplanin expression in osteocytes and osteoblasts [32], odontoblasts and enamel epithelia [27], mesothelial cells [19], epidermal basal layer cells [12], choroid plexus epithelial cells [34], thymus type I epithelial cells [3], prostate myofibroblasts [20, 24], follicular dendritic cells [14, 35], and immature cells like fetal germ cells and developing Sertoli cells [10, 29, 37]. Elucidation of the biological importance of these somatic tissues is required. We were initially interested in examining the lymphatic distribution in major salivary grands by immunostaining for a lymphatic endothelial cell marker, podoplanin. By chance, we discovered that most podoplanin-positive cells in major salivary glands were myoepithelial cells rather than lymphatic endothelial cells [8]. Podoplanin expression is rarely found in the acini of the parotid gland but clearly found at the basal portion surrounding acinar cells in submandibular and sublingual glands. Strong expression of podoplanin is also found at the basal portion of the intercalated duct, striated duct, and interlobular duct in all major salivary glands [8]. The terminal portion of the parotid gland is covered by only a few myoepithelial cells whereas the terminal portions of sublingual and submandibular gland, and also the ducts are covered by a number of broad and extensive cellular processes [18]. Podoplanin could be a marker protein of salivary gland myoepithelial cells because podoplanin detection levels coincide with the distribution of myoepithelial cells specific for major salivary glands. In a previous study, podoplanin-positive cells were examined in connection with the reaction with the antibody to the mammary gland myoepithelial marker P-cadherin [8]. The elucidation of podoplanin localization on the myoepithelium requires a transmission immunoelectron microscopic study. However, the expression of podoplanin in salivary glands has not been examined at the electron microscopic level. This study was aimed to investigate the immunoreaction of podoplanin-positive cells with α-smooth muscle actin (SMA) used as a marker for myoepithelial cells, and to elucidate podoplanin localization in salivary gland myoepithelial cells by the immunoelectron microscopic study.

II. Materials and Methods

Immunostaining for fluorescence microscopy

The salivary gland tissue of 8-week-old closed colony ICR mice (male, n=5; Charles River Japan Inc., Yokohama, Japan) was used. Tissue was obtained after euthanasia by intraperitoneal injection with sodium pentobarbital (10 ml/kg, Nembutal, Abbott Laboratories, North Chicago, IL, USA). The protocol for animal use was reviewed and approved by the animal experiment committee of Fukuoka Dental College, Fukuoka, Japan. We initially used glutaraldehyde and formaldehyde to fix tissue, but when the tissue was treated by the solution this extremely reduced the immunoreactivity of an antibody for podoplanin. Therefore, the present study used frozen 8 μm sections which were cut in a cryostat and fixed in 100% methanol for 2 min at –20°C for the immunofluorescent staining and the pre-embedding immunoelectron microscopic examination. After treatment with 0.1% goat serum (GS) diluted with 10 mM phosphate-buffered saline (PBS, pH 7.4) for 10 min at room temperature (RT), sections were treated with a cocktail of primary antibodies: 1 μg/ml of monoclonal hamster anti-mouse podoplanin (anti-podoplanin; AngioBio Co., Del Mar, CA) and monoclonal rabbit anti-α-smooth muscle actin (anti-α-SMA; Epitomics, Inc., Burlingame, CA) for 8 hr at 4°C. Sections were reacted with a cocktail of second antibodies: 1 μg/ml of AlexaFluor 488-conjugated goat anti-hamster IgG and AlexaFluor 568-conjugated goat anti-rabbit IgG (Molecular Probes, Invitrogen, Eugene, OR) in GS-PBS for 1 hr at RT, and then examined by a fluorescence microscopy BZ-8100 (Keyence Corp., Osaka, Japan).

Furthermore, the tissue was trimmed on small pieces of the 5 mm width, fixed in 100% methanol for 10 min at –20°C, treated with GS-PBS for 3 hr at RT, and then directly immersed in a cocktail of first antibodies: 1 μg/ml of anti-podoplanin and anti-α-SMA for 8 hr at 4°C. Next, the tissue was exposed to a cocktail of second antibodies: 1 μg/ml of AlexaFluor 488-conjugated goat anti-hamster IgG and AlexaFluor 568-conjugated goat anti-rabbit IgG in GS-PBS for 2 hr at RT. The trimmed tissue was also exposed to 1 μg/ml of anti-podoplanin and treated with a cocktail of 1 μg/ml of AlexaFluor 488-conjugated goat anti-hamster IgG and rhodamine-conjugated concanavalin A (Con A; Molecular Probes) in PBS to visualize the cell membrane. The immunostained tissues were examined by laser-scanning confocal microscopy (Axiovert 135M, Carl Zeiss, Jena, Germany) with a ×40 oil Plan-Apochromatic oil immersion objective lens (numerical aperture ×1.3).

Immunostaining for electron microscopy

The present study used pre-embedding immunoelectron microscopy. The tissue was trimmed to small pieces of 5 mm width. Frozen 40 μm sections were cut in a cryostat and fixed in 100% methanol for 10 min at –20°C. After treatment with GS-PBS, sections were treated with anti-podoplanin (10 μg/ml; AngioBio Co., Del Mar, CA) for 8 hr at 4°C. Next, sections were reacted with a 5 μg/ml of rabbit anti-goat IgG (Vector-ABC kit; Vector Laboratories, Inc., Burlingame, CA) for 2 hr at RT and with DAB substrate kit (Vector) for 1 hr at RT, and were post-fixed by a 4% paraformaldehyde-PBS for 8 hr at RT. Sections were sequentially fixed in 1% osmium tetroxide-PBS for 30 min at RT, dehydrated in an ascending ethanol series, and embedded in a resin mixture (Epon 812, 4.8 g; DDSA EM grade, 1.9 g; MNA EM grade, 3.3 g; DMP-30, 2 g). Ultrathin sections were prepared with a ultramicrotome (Reichert-Nissei Ultracuts; Leica Microsystems GmbH, Wetzlar, Germany) with a diamond knife, exposed to 5% uranyl ace-

tate in 50% ethanol for 15 min at RT, washed by deionized water (DW), exposed to 0.4% lead citrate and 0.4% sodium hydroxide in DW for 5 min at RT, and examined under a transmission electron microscope (JEM-1400, JEOL Ltd., Tokyo, Japan).

III. Results and Discussion

Immunohistochemistry for the expression of podoplanin and α-SMA in salivary glands

Myoepithelial cells exist around acinar cells and at the basal side of ducts in salivary glands [18]. Myoepithelial cell shape varies considerably with different glands in relation to the physical properties of secretions. In the parotid gland, few myoepithelial cells are present on serous acini except for ducts while in the sublingual gland, approximately 50% of the terminal portion surfaces of mucous acini are covered by a number of broad and extensive myoepithelial cell processes [18]. Therefore, in the present study, the congruity of the podoplanin-positive region to the region where the well-established myoepithelial cell marker α-SMA is positive was investigated by whole staining because myoepithelial cells exist on the surface of acini and ducts [1, 2, 6, 13, 21]. Furthermore, the cross reaction of anti-podoplanin was investigated by tissue section because no myoepithelial cells exist in the center of acini and the luminal side of ducts [18]. As for the whole staining of the sublingual glands, the surfaces of the whole terminal portion of mucous acini were stained by a universal cell membrane marker, Con A. Terminal portion surfaces were also covered by a number of extensive myoepithelial cellular processes and the immunostaining level by anti-podoplanin to myoepithelial cells on terminal portion surfaces completely coincided with the immunostaining by anti-α-SMA (Fig. 1). These findings suggest that podoplanin is an antigen for salivary gland myoepithelial cells, and that the immunostaining of podoplanin in the salivary glands directly reflects myoepithelial cell shapes. For the immunostaining of salivary gland tissue sections, the region around the acinar cells was immunostained by both anti-podoplanin and anti-α-SMA. In the sublingual mucous acini there was more region immunostained with anti-podoplanin in the sublingual gland mucous acini than in the parotid gland serous acini. The basal side of the ducts was also stained with anti-podoplanin in both the parotid and sublingual glands at the same levels (Fig. 2). These findings suggest that the detection levels of podoplanin in the salivary glands reflect the distribution of myoepithelial cells. The basal side of the ducts was stained with anti-podoplanin

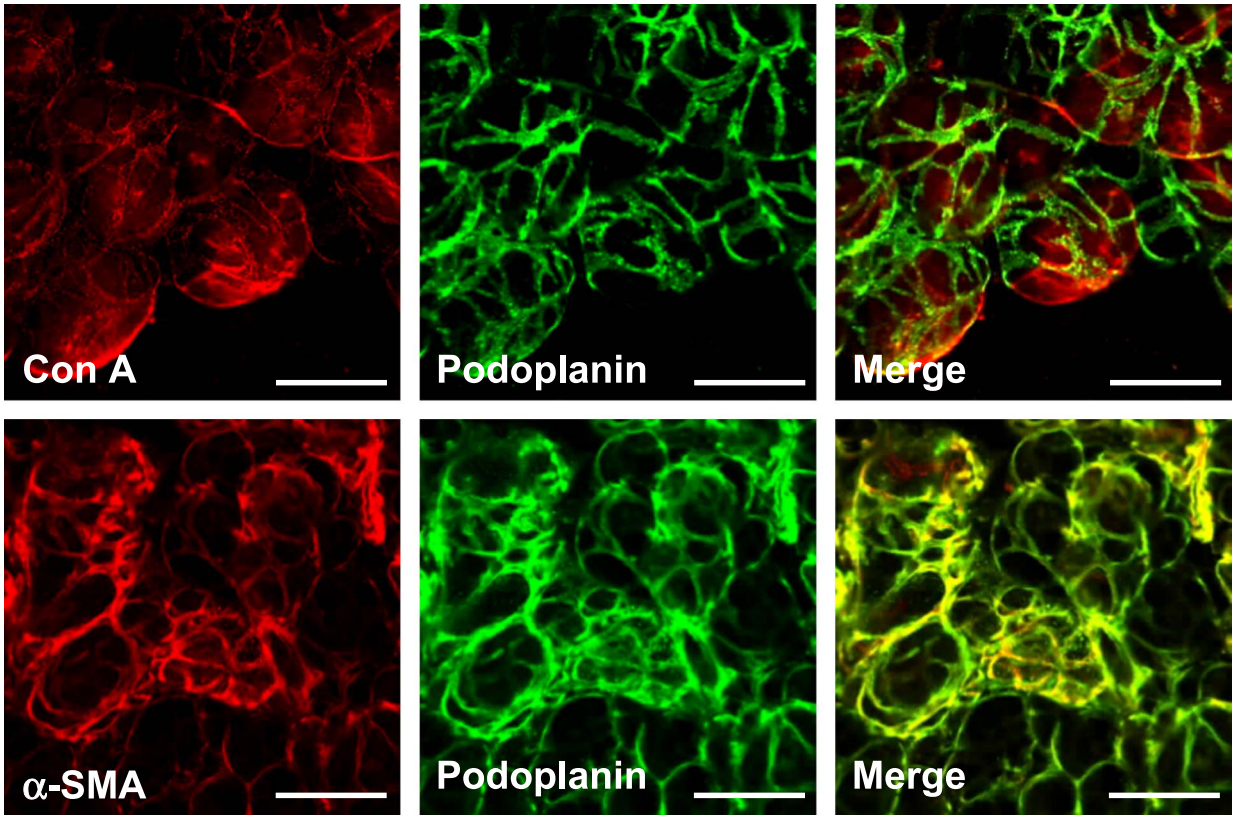

Fig. 1. Whole staining of sublingual gland with anti-podoplanin and anti-α-SMA. Concanavalin A (Con A) was used to visualize the cell membrane of the acinar terminal portion in red. Terminal portion surfaces of mucous acini are covered by a number of extensive myoepithelial cellular processes with green fluorescence immunostaining by anti-podoplanin of which the degree and extent completely coincide with anti-α-SMA immunostaining. Bar=100 μm.

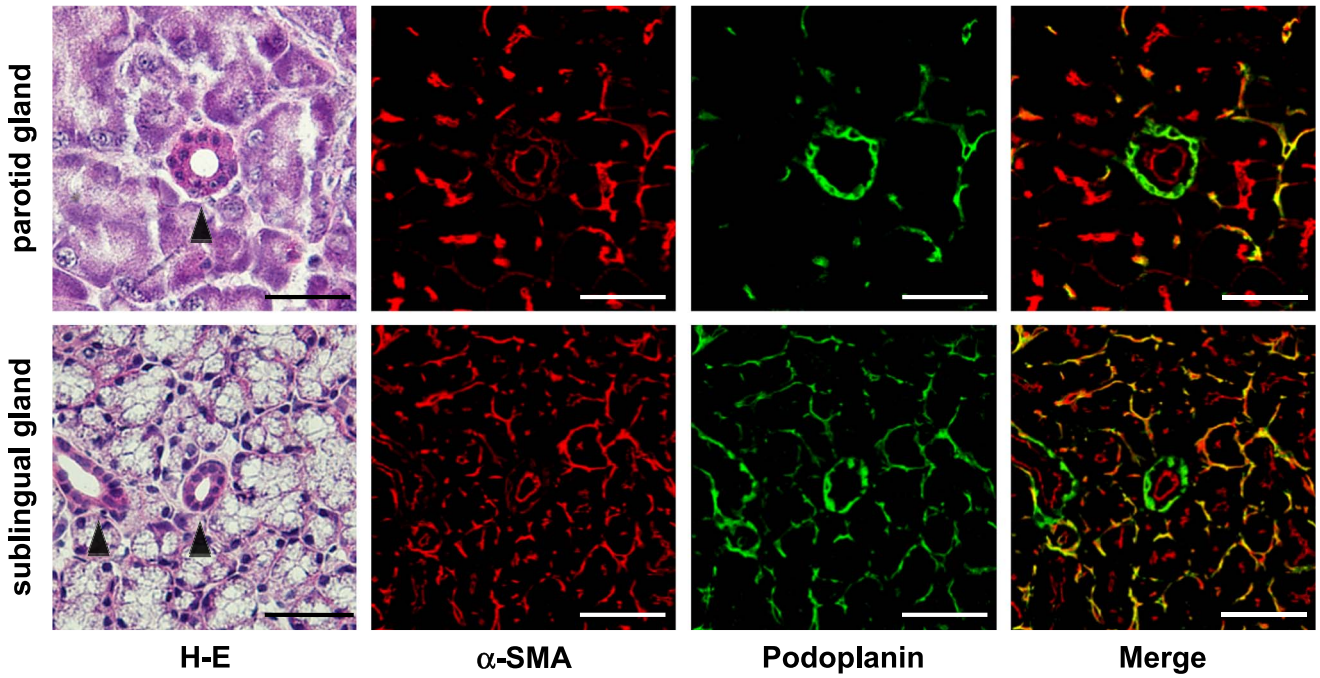

Fig. 2. Comparison of immunostaining by anti-podoplanin and anti-α-SMA between parotid and sublingual glands. In mouse parotid glands the terminal secretory end pieces of acini were densely composed of serous pyramidal cells with basophilic cytoplasm. In sublingual gland the terminal secretory end pieces of acini were composed of large mucus-filled cells with cytoplasm which was less basophilic than that of the parotid glands. The portion around the acinar cells is immunostained by both anti-podoplanin and anti-α-SMA (merged, in yellow). In the sublingual gland mucous acini there are more parts that are immunostained with anti-podoplanin in the sublingual gland mucous acini than in the parotid gland serous acini. The basal side of the ducts is also immunostained with anti-podoplanin in both the parotid and sublingual glands at the same level, and to a stronger extent than with anti-α-SMA (arrowheads, intercalated duct). There are more parts that are immunostained with anti-α-SMA than with anti-podoplanin. Reaction products with anti-α-SMA are also found in the center of the acinar and at the luminal side of the duct where reaction products with anti-podoplanin are not found. Bar=100 μm.

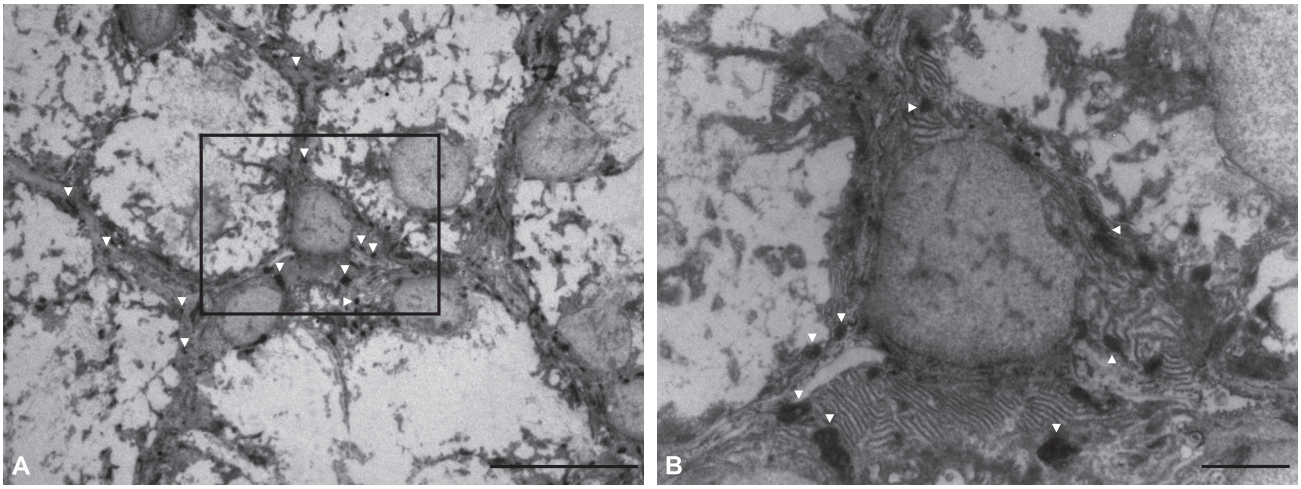

Fig. 3. Immunoelectron microscopy for podoplanin production in the cytoplasm of salivary gland myoepithelial cells. (**A**) A strong density of immunostaining with anti-podoplanin is found in the region of the cell membrane and in the cytoplasm of myoepithelial cells between sublingual gland acinar cells. Some reaction products of anti-podoplanin are also observed at the basal side in the cytoplasm of the acinar cells. Bar=10 μm. (**B**) In the higher magnification of the cell highlighted in **A** (box), a number of reaction products with anti-podoplanin are found at the Golgi apparatus binding to endoplasmic reticulum (arrowheads). Bar=2 μm.

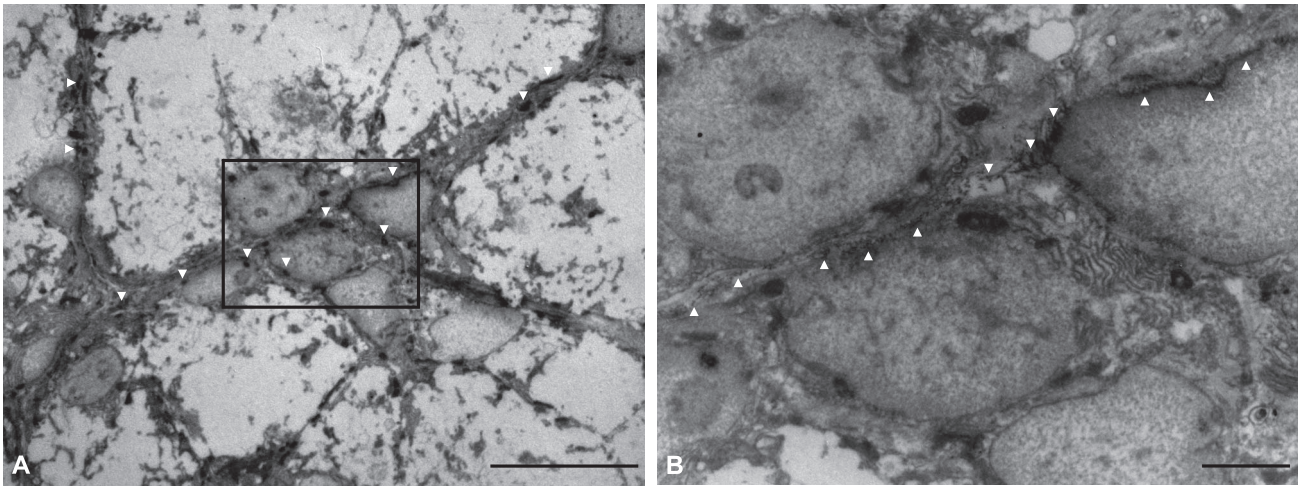

Fig. 4. Immunoelectron microscopy for the membrane localization of podoplanin in salivary gland myoepithelial cells. (**A**) A strong density of immunostaining with anti-podoplanin is found at the cell membrane and in the cytoplasm of myoepithelial cells between sublingual gland acinar cells. Bar=10 μm. (**B**) A number of small DAB granules with strong density by immunostaining with anti-podoplanin are found at the myoepithelial cell membrane between sublingual gland acinar cells (arrowheads). Bar=2 μm.

to a stronger extent than with anti-α-SMA in both parotid and sublingual glands (Fig. 2), suggesting that salivary gland myoepithelial cells may be more abundant in podoplanin than in α-SMA. On the other hand, the center of acini and luminal side of ducts were immunostained with anti-α-SMA but not by anti-podoplanin (Fig. 2). Since these are regions without salivary gland myoepithelial cells [18], it was conjectured that anti-α-SMA caused cross reaction to the actin present in cells universally.

Immunoelectron microscopy for the expression of podoplanin in salivary gland myoepithelial cells

A number of reaction products with anti-podoplanin were found at the Golgi apparatus binding to endoplasmic reticulum in the cytoplasm of myoepithelial cells between sublingual gland acinar cells (Fig. 3). Furthermore, a number of reaction products with the anti-podoplanin were localized at the myoepithelial cell membrane between acinar cells (Fig. 4). The Golgi apparatus is composed of membrane-bound cisternae which have networks to the endoplasmic reticulum, and primarily functions to process proteins, package the macromolecules, and enable them to make their way to their destination [23]. The Golgi apparatus plays a role in the attachment of polysaccharides to a protein synthesized in the endoplasmic reticulum to form proteoglycans. Podoplanin is a mucin-type transmembrane glycoprotein negatively charged by extensive O-glycosylation and has a high content of sialic acid. Therefore, it is thought that podoplanin is continuously produced, glycosylated with polysaccharides in the Golgi apparatus, and transported to the cell membrane. Some reaction products with anti-podoplanin were observed at the basal side near the nuclei in the cytoplasm of the acinar cells in spite of the absence of reaction products with anti-podoplanin in the salivary gland acinar cells by fluorescence microscopy. Further studies on podoplanin localization in acinar cells by immunoelectron microscopy are required. It has been reported that podoplanin promotes plasma membrane extension and actin cytoskeleton rearrangement [15, 30, 33, 36], and that podoplanin is resistant to proteases because it is a negatively charged mucin-type protein [3]. Podoplanin may play a role in maintaining the shape of myoepithelial cell foot processes to protect acinar cells from the outside.

IV. References

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