

Morphological and Functional Analyses of the Tight Junction in the Palatal Epithelium of Mouse

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Tight junction (TJ) is one of the cell-cell junctions and known to have the barrier and fence functions between adjacent cells in both simple and stratified epithelia. We examined the distribution pattern, constitutive proteins, and permeability of TJ in the stratified squamous epithelium of the palatal mucosa of mice. Ultrastructural observations based on the ultrathin section and freeze-fracture methods revealed that poorly developed TJs are located at the upper layer of the stratum granulosum. The positive immunofluorescence of occludin (OCD), claudin (CLD)-1 and -4 were localized among the upper layer of the stratum granulosum showing a dot-like distribution pattern. And CLD-1 and -4 were localized among the stratum spinosum and the lower part of stratum granulosum additionally showed a positive reaction along the cell profiles. Western blotting of TJ constitutive proteins showed OCD, CLD-1, -2, -4, and -5 bands. The permeability test using biotin as a tracer revealed both the areas where biotin passed through beyond OCD positive points and the areas where biotin stopped at OCD positive points. These results show that poor TJs localize at the upper layer of the stratum granulosum of the palatal epithelium, and the TJs are leaky and include at least CLD-1 and -4.

Key words: tight junction, palatal epithelium, claudin, occludin, stratum granulosum

I. Introduction

The epithelium is a continuous sheet of cells that covers the external surface of the body, as well as lines the internal cavities and organs. A simple epithelium consists of four types of cell junctions, namely tight junctions (TJs), adherence junctions, desmosomes and gap junctions at the

lateral cell membranes of adjoining cells. Among these cell junctions, TJs are located at the most apical site of the lateral membranes of simple epithelial cells. Farquhar and Palade [8] first reported the fine structural feature of a TJ showing that the outer leaflets of cell membranes of adjoining cells were fused. Nowadays it has been recognized that these TJs are observed as a continuous branching network showing strands on the protoplasmic face (P-face) and grooves on the corresponding extracellular face (E-face) by the freeze-fracture method [29]. The functions of TJs have been thought to be a sealing structure that regulates the paracellular transport of materials, and also works as a fence to maintain cell polarity by preventing membrane fluidity [25]. With regard to the protein compositions of

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TJs, the adhesion sites consist of the transmembrane proteins, occludin (OCD) and claudins (CLDs) [10, 11]. OCD was discovered as a TJ protein and is assumed to exist universally in TJs [10]. On the other hand, CLD forms a multi-gene family of 27 members in mammals [20] and shows that tissue and cell-specific expression varied significantly depending on CLD species [21].

In the stratified epithelia, TJs have been reported by Hashimoto [13], and Shimono and Clementi [26] to exist in the epidermis and the oral epithelium, respectively. Stratified epithelia consist of two or more layers of cells and their main function is to provide protection by forming a barrier. However, the barrier function in the epithelia had been neglected in the past decades [2, 6]. Furuse *et al.* [12] put an end to the debate, that is, they clearly proved the existence of functional TJs in mouse skin epidermis using molecular biology, immunohistochemistry, and electron microscopy.

Oral epithelium, which consists of a stratified squamous epithelium, is exposed to severe conditions such as mechanical stimulus during mastication and alien substances. The TJs in the oral mucosa have been demonstrated in the gingiva by electron microscopic observations [17], and CLD compositions in the gingiva and the buccal mucosa [1, 15, 24]. However, no report is available with regard to TJ associated barrier function of oral epithelia. The purpose of the present study was to examine the barrier function of oral epithelium employing palatal mucosa classified as a masticatory mucosa, with special reference to TJ distribution patterns by electron microscope, OCD and CLDs localizations and permeability of the epithelial TJ.

II. Materials and Methods

Eight-week-old male mice (ICR strain) were obtained from the CLEA Japan, Inc. (Tokyo, Japan). The palatal mucosa was collected. All experimental procedures have been authorized by the Animal Care and Use Committee, Okayama University (OKU-2015097).

Electron microscopic analyses

1) Analysis by ultrathin sections

The ultrathin sections of palatal epithelia were observed employing both conventionally prepared specimens and lanthanum impregnated specimens to identify TJs. The sections were stained with uranium acetate and lead citrate for observation.

a) Conventional method

The mice were anesthetized and perfused via the left ventricle with 2% glutaraldehyde and 2% paraformaldehyde mixture in 0.05 M phosphate buffer (pH 7.4) for 5 min. Then the palate was removed and additionally fixed in the same fixative for 24 hr. After the fixation the specimens were decalcified with 0.05 M phosphate buffered 5% ethylenediaminetetraacetic acid (EDTA) (pH 7.4) for 3–4 days, post-fixed with 0.05 M phosphate (pH 7.4) buffered

1% osmium tetroxide, which was preceded by rinsing with the buffer solution for several hours, dehydrated with a graded acetone series, and embedded in Epon 812.

b) The lanthanum impregnation method

Lanthanum impregnated specimens were prepared to clearly identify TJs based on the method described by Hashimoto [13]. Mice were sacrificed, and palatal mucosae were removed from the palate with a metal spatula. After the specimen collection, the impregnation process was conducted. In brief, the specimens were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) containing 1% lanthanum nitrate for 2 hr, washed for 12 hr, and then finally post-fixed in 1% osmium tetroxide in the same buffer for 2 hr. The specimens were dehydrated with a graded series of ethanol containing 1% lanthanum nitrate and embedded in Epon 812.

2) Analysis by the freeze-fracture method

Palates from the mice were collected after perfusion-fixation with 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4), additionally fixed for 24 hr, decalcified with EDTA as mentioned above, cut into small pieces, washed for 12 hr in the 0.05 M phosphate buffer (pH 7.4), and immersed in 30% glycerin solution to prevent ice crystal formation. The specimens were frozen with liquid nitrogen. They were immediately transferred into a freeze-fracture device (BAF060; Bal-Tek, Hudson, NH), fractured at -110°C shadowed with platinum at an angle of 60° following the evaporation of carbon at an angle of 90° .

Immunofluorescent microscopy

For immunohistochemical detection of TJ-associated proteins, polyclonal goat antibodies against OCD (Santa Cruz Biotechnology, Dallas, TX), polyclonal rabbit antibodies against CLD-1 (Thermo Fisher Scientific, Waltham, MA), CLD-2 (Abcam, Cambridge, England), CLD-3 (Thermo Fisher Scientific), CLD-4 (Thermo Fisher Scientific), and CLD-5 (Abcam) were employed. As secondary antibodies, rabbit anti-goat IgG labeled with Alexa Fluor 488 (Abcam) or goat anti-rabbit IgG labeled with Alexa Fluor 488 (Thermo Fisher Scientific) were used based on the primary antibodies applied.

The mice were decapitated after anesthesia. The heads were immediately frozen in isopentane cooled with liquid nitrogen. Five-micron sections were made in a cryostat according to the method introduced by Kawamoto [16]. The cut sections were fixed in 95% ethanol at 4°C for 30 min and then immersed in 100% acetone for 1 min at room temperature. They were soaked in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 1 hr, and then incubated with 1:100–200 diluted primary antibodies for 4 hr. The incubation was terminated by PBS. The secondary antibodies labeled with Alexa Fluor 488 were applied for 1 hr. The incubation was conducted at room temperature. Nuclear staining was performed with Hoechst33342 (Thermo Fisher Scientific) for 10 min as

occasion demanded. Control sections were incubated without primary antibodies for OCD, CLD-1 and -4, respectively. The slides were sealed with VECTASHIELD (Vector Laboratories, Burlingame, CA).

Western blotting

The palatal mucosae removed were saved in radio-immunoprecipitation assay buffer containing 1% protease inhibitor, and the tissue were homogenized in PBS containing 0.5% sodium dodecyl sulfate (SDS) and 2% mercapto-ethanol. Following centrifugation at $12,000 \times g$ for 10 min at 4°C, supernatants were collected and then gels were electroblotted on polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). After transblotting, OCD, CLD-1, -2, -3, -4 and -5 expressions were examined. The antibodies used for western blotting were the same as that mentioned in the immunohistochemical method.

Barrier function assay

To assess the barrier function of the TJs in the palatal mucosa, the surface biotinylation technique was applied based on the method developed by Chen *et al.* [3]. EZ-Link Sulfo-NHS-LC-Biotin (50 μ l of 10 mg/ml; Thermo Fisher Scientific) in PBS containing 1 mM CaCl_2 was injected into the submucosal tissue of the palate. After 30 min, the palatal mucosa was collected and frozen in isopentane cooled with liquid nitrogen. Five micron frozen sections were cut in a cryostat, fixed in 95% ethanol at 4°C for 30 min, and then immersed in 100% acetone at room temperature for 1 min. The sections were rinsed in 1% BSA in PBS for 1 hr, and then incubated with 1:100 diluted polyclonal goat antibodies against OCD for 4 hr at room temperature. After incubation, secondary antibody (rabbit anti-goat IgG labeled with Alexa Fluor 488) was applied for 30 min at room temperature, and then incubation with Streptavidin Alexa Fluor 594 conjugate (Thermo Fisher Scientific) was conducted for 30 min at room temperature. The sections were nuclear stained by Hoechst33342 and mounted with VECTASHIELD.

III. Results

Electron microscopic observations

The palatal epithelium was confirmed to consist of stratum basale, stratum spinosum, stratum granulosum, and stratum corneum by semi-thin sections (Fig. 1). TJs were rarely observed at the upper layer of stratum granulosum by conventional and lanthanum impregnation methods (Fig. 2A, B), but were not confirmed at other layers including the lower layer of stratum granulosum. In the freeze-fracture method, TJ strands were clearly revealed at the similar area of the stratum granulosum as observed by thin sections, but not at other layers. The TJ strands observed were quite few in number, and each strand discontinuously ran with relatively wide gaps (Fig. 2C).

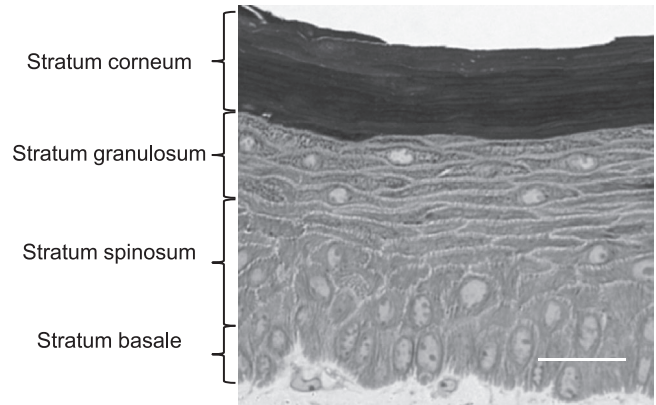


Fig. 1. Structure of the mouse palatal epithelium. The palatal epithelium is composed of stratum basale, spinosum, granulosum, and corneum. The stratum basale was observed as a single layer, and the stratum spinosum consisted of several layers of cubic or cylindrical cells. The stratum granulosum showed flattened cell layers, and included fine granules in the cytoplasm. The stratum corneum was relatively thick. A toluidine blue stained semi-thin section. Bar = 20 μ m.

Immunofluorescent microscopy

In the epithelium of the palatal mucosa, the positive reactions were detected for OCD, CLD-1 and -4 (Fig. 3), and the distribution patterns of these positive sites were different. The OCD positive reaction products were sparsely distributed showing dot-like fluorescence, and they were restricted between the upper layers of stratum granulosum (Fig. 3A). CLD-1 and -4 showed the same distribution patterns of the reaction products as the OCD on the upper layers of stratum granulosum, however these two claudins additionally showed positive reaction along the cell profiles showing linear reactivity in the stratum spinosum and the lower layers of the stratum granulosum (Fig. 3C, D). Although CLD-4 showed the same pattern as the CLD-1, the network-like positive range was slightly narrow in comparison to CLD-1. CLD-2, -3, and -5 were negative, and the stratum basale showed no reaction in the TJ associated proteins examined in the present study. CLD-2 and CLD-5 were not detected in the epithelium, but positively stained in the salivary glands and blood vessels, respectively (Fig. 3F, G). The controls for OCD, CLD-1 and -4 incubated without primary antibodies were negatively stained (Fig. 3B, E).

Western blotting

The palatal mucosa collected included stratified squamous epithelium, and proprial layer that contained palatine glands and blood vessels. Employing these specimens, western blotting clearly showed the existence of OCD, CLD-1, -2, -4, and -5 in the palatal mucosa, but not of CLD-3 (Fig. 4).

Barrier function assay

It was found that the biotin injected into the submucosal connective tissue as a tracer passed through the

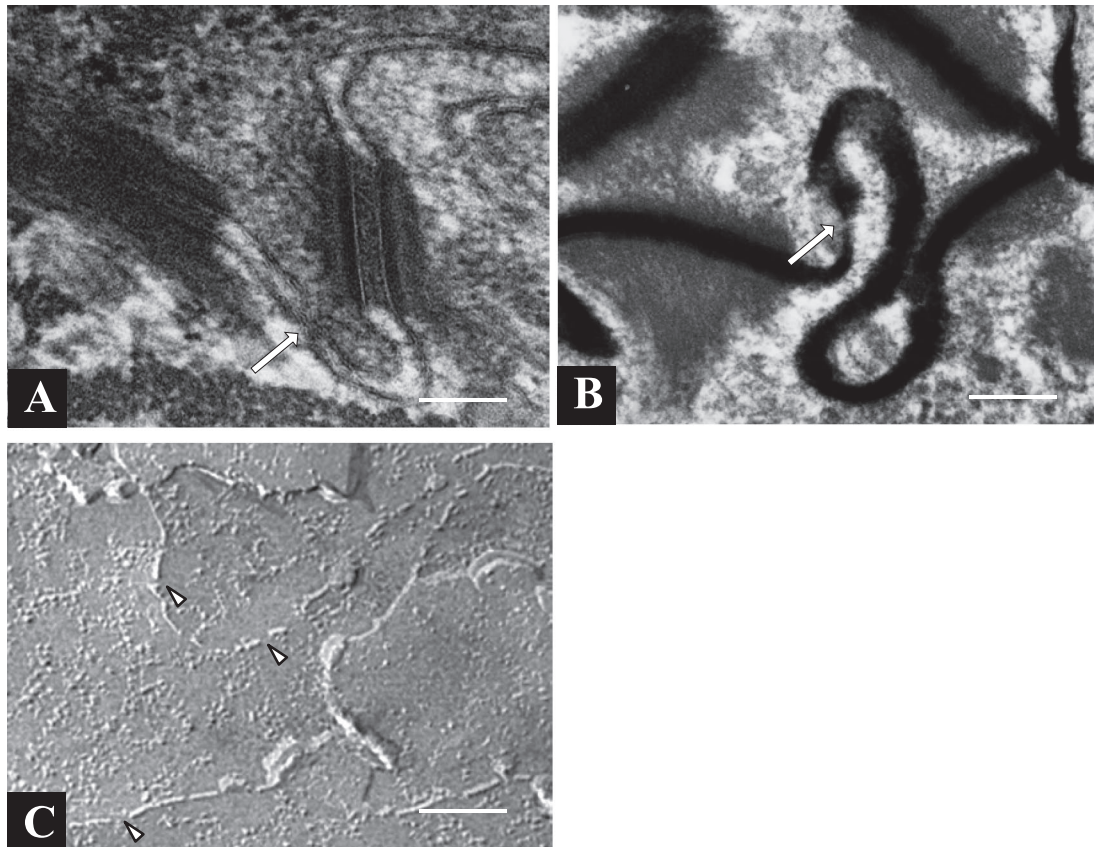


Fig. 2. Electron micrographs at the upper layer of stratum granulosum. (A) TJ is shown as a cell membrane fused point (an arrow) in the stratum granulosum by conventional electron microscopic observation. A thin section stained with uranium and lead citrate. (B) Lanthanum nitrate penetrated the intercellular spaces between neighboring cells of the stratum granulosum. TJ was identified by the interruption of lanthanum penetration (an arrow). The lanthanum impregnation method. (C) The freeze-fracture method revealed TJ strands (arrowheads) on the P face. The strands were poorly distributed, and the discontinuous sites of strands were frequently observed. Bars = 250 nm.

intercellular space of the epithelial cells toward the direction of the stratum corneum. The double stain of the biotin and the OCD showed two patterns, i.e. the permeated biotin stopped at the point harmonized with the OCD positive points and the biotin penetrated beyond the OCD positive points (Fig. 5).

IV. Discussion

It is generally accepted that TJs are located at the most apical region between simple epithelial cells and have a barrier function that controls a paracellular transport of the substance. In addition to simple epithelia, Furuse *et al.* [12] demonstrated that the TJs in the stratified squamous epithelium in the epidermis and stated that they distribute in the apical few cell layers of the stratum granulosum and function as a barrier against water transpiration through the skin. In oral epithelia, however the existence of TJs was detected by means of ultrathin sections, but the functional significance was doubtful because the frequency of the TJs was quite low [23, 26, 27]. On the other hand, OCD is a reliable marker protein for TJ [12] and this was observed in oral mucosae, namely the buccal mucosa in dogs [1] and

the gingiva in humans [5, 15, 18], and pigs [24]. However, the distribution pattern and barrier ability in oral epithelia are still unclear, and no report is available for TJs in the palatal epithelium. In the present study, however, the frequency was also low, the TJs were confirmed at the apical region (about one third) of the stratum granulosum by the conventional and the lanthanum impregnated ultrathin sections, and the immunofluorescence of the OCD. In addition, the freeze-fracture replica method clearly showed few TJ strands in the area observed by the methods mentioned above. These results are the first reported by any study. Furthermore, the results indicated that the TJs in the palatal epithelium were poorly developed because the TJ strands were few in number and discontinuous sites frequently appeared in the strands.

In oral mucosae, OCD, and CLD-1, -4 and -7 have been detected in the buccal epithelium, and the outer and inner epithelia of gingiva in some mammalian species [1, 5, 9, 15, 24]. However the combinations of CLD expression patterns vary depending on the types of mucosae and species. In the present study, CLD expressions (CLD-1 to -5) were examined using both immunohistochemistry and western blotting. Immunohistochemistry positively showed

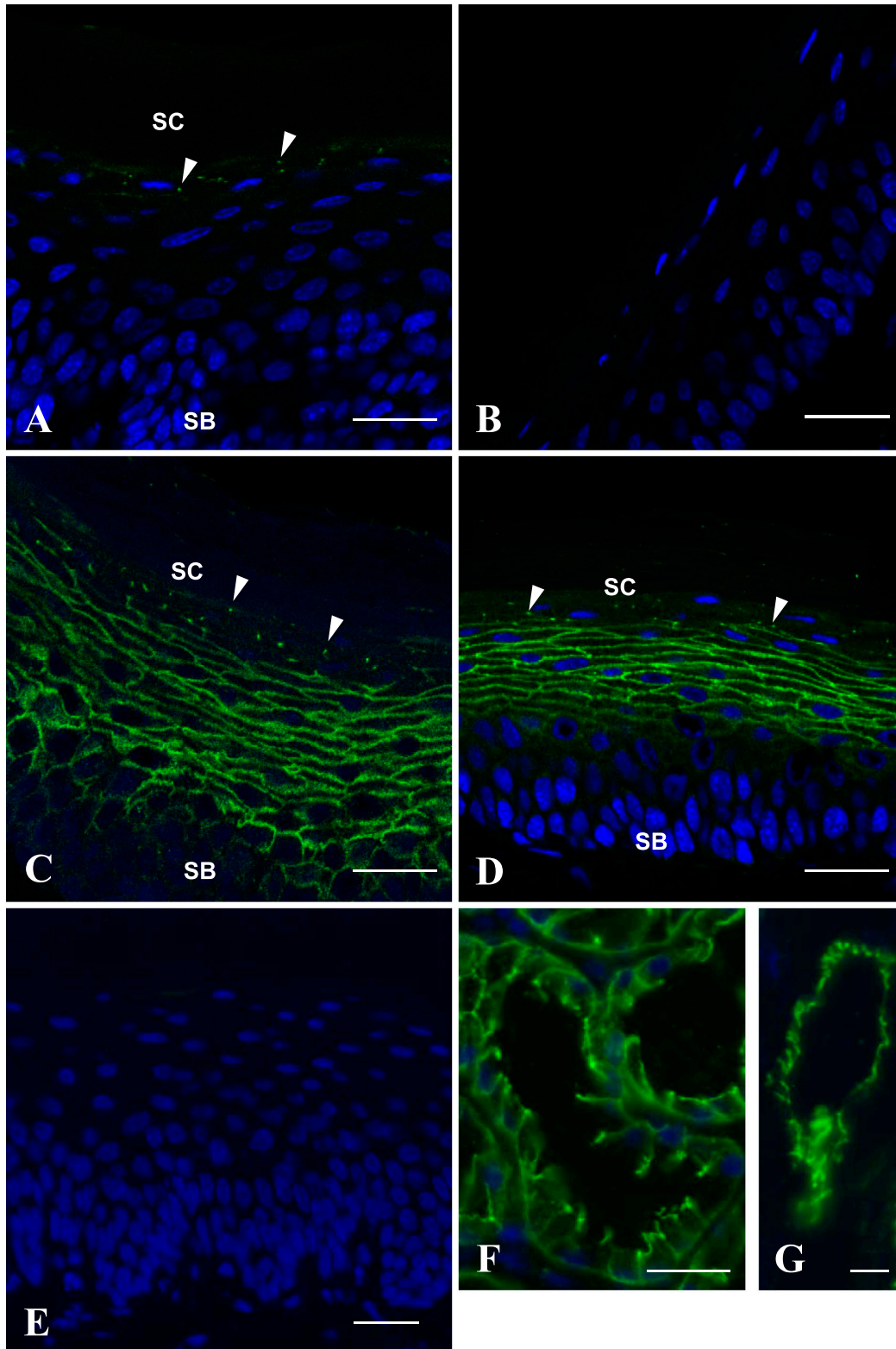


Fig. 3. Immunohistochemistry of TJ constitutive proteins. The immunofluorescence of OCD (A), CLD-1 (C), and -4 (D) were localized between the upper layers of the stratum granulosum showing dot-like fluorescence (arrowheads). In addition, CLD-1 (C) and CLD-4 (D) showed network-like positive reaction from the lower layer of the stratum granulosum to the upper layer of the stratum spinosum. CLD-2 (F) and CLD-5 (G) were positive in the salivary glands and the endothelium of blood vessel respectively, but were negative in the palatal epithelium. Background staining levels were checked by omitting the primary antibodies (B: rabbit anti-goat IgG labeled with Alexa Fluor 488, E: goat anti-rabbit IgG labeled with Alexa Fluor 488). SC: stratum corneum, SB: stratum basale, Blue: nuclei stained with Hoechst. Bars = 25 μm (A, B, C, D, E), 50 μm (F), 10 μm (G).

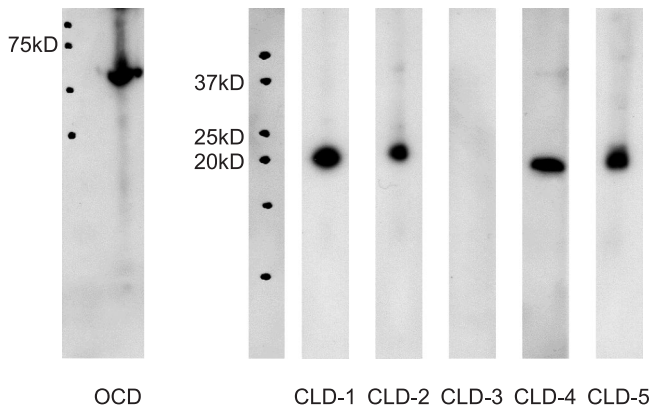


Fig. 4. Western blotting of the palatal mucosa. OCD, CLD-1, -2, -4, -5 were positive, but CLD-3 was negative in the palatal mucosa.

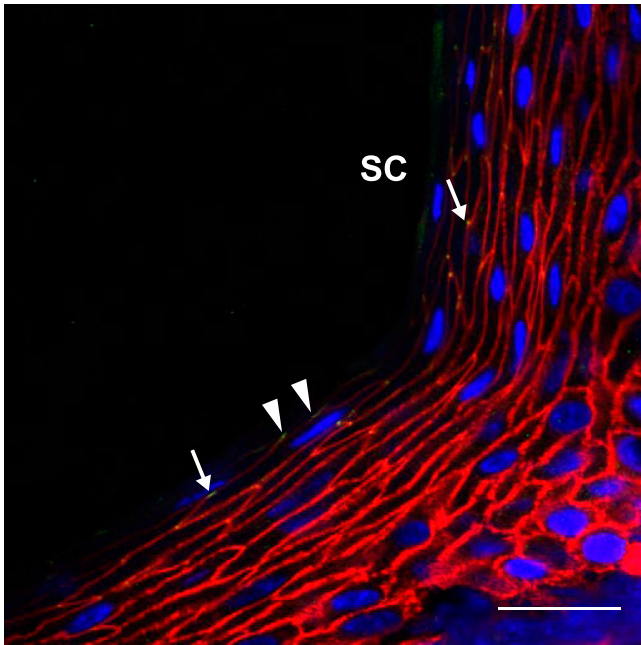


Fig. 5. Permeability of the palatal epithelium with the use of biotin as a tracer. Biotin penetrated between the intercellular spaces from the stratum basale toward the stratum corneum in the palatal epithelium. The sites where biotin was stopped at the OCD positive sites (arrowheads) were observed. On the other hand, the sites where biotin passed through the OCD positive sites were also shown (arrow). SC: stratum corneum, Red: biotin, Green: OCD, Blue: nuclei. Bar = 25 μ m.

CLD-1 and -4 in the epithelium. The other CLDs were negative. Western blotting detected the bands of CLD-1, -2, -4, and -5, but negative for CLD-3. That is, CLD-2 and -5 were positive in western blotting but negative in immunohistochemistry. These two CLDs might be derived from the TJs of minor salivary glands [14, 19] and blood vessels [19, 22] existed in the proprial layer because the layer was included when the specimens were collected for western blotting. Therefore, it was shown that CLD-1 and -4 existed together

with OCD in the palatal epithelium by immunohistochemistry and western blotting in the present study. The result obtained is in concurrence with the epidermis of the skin [12]. It would be worthwhile to examine other types of CLDs in the palatal epithelium because CLDs comprise a family consisting of 27 members [20].

With regard to the distribution patterns of CLD-1 and -4 in the palatal epithelium, CLD-1 and -4 were positively stained throughout the stratum granulosum, and the former was also positive in the whole stratum spinosum, but the latter was positive in the upper half of the layer. The fluorescence was observed along the cell profiles at the positive cells. TJs did not exist in these cell layers by fine structural observations and the immunohistochemistry of OCD. In the present study, we could not understand the significance about the existence of CLD-1 and -4 where the TJs were not there. We can only point out this interesting phenomenon.

Regarding the barrier function of TJs, it has been reported that the barrier function is strengthened depending upon the number of TJ strands [4]. Elias *et al.* [7] reported that with the use of the freeze-fracture method, the stratified squamous epithelia in skin, esophagus, and vagina consisted of only 1 to 3 strands, so that the barrier function in these epithelia was assumed to be low. On the other hand Furuse *et al.* [12] elucidated that the epidermal TJs functioned against water transpiration through the skin and that the CLD-1 expressed in epidermal TJs was essential for the function using CLD-1-deficient mice and a tracer experiment. No report is available with regard to the functional evaluation of the TJs in the palatal epithelium. Surface biotinylation technique developed by Chen *et al.* [3] was employed to estimate the paracellular permeability in the present study. The biotin injected penetrated the intercellular spaces and the penetration was stopped at the OCD positive points. On the other hand, biotin was also observed beyond the OCD positive points. These results suggest that the barrier ability by the TJs in the palatal epithelium is not so firm, but leaky. This point of view is also supported by the freeze-fracture experiment in the present study.

In the present study, it was shown that leaky TJs existed and was suggested that the TJs consisted of at least CLD-1 and -4 in addition to OCD in the palatal epithelium. In fact, a physiological report indicates that water and proteins easily permeate palatal mucosa in comparison to the skin [28]. It would be necessary to evaluate the functional significance of the TJs in the palatal epithelium as a barrier apparatus.

V. Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

VI. References

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