Tight Junction Transmembrane Protein Claudin Subtype Expression and Distribution in Human Corneal and Conjunctival Epithelium

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PURPOSE. The combination of the tight junction transmembrane protein claudin subtypes is one of the most important determinants of variations in the tightness of individual paired tight junction strands. The barrier function of corneal epithelium is much stronger than that of conjunctival epithelium. In this study, the expression and cellular distribution of claudin species in in vivo human corneal and conjunctival epithelium were investigated.

METHODS. Reverse transcription-polymerase chain reaction was used to reveal the claudin mRNA. Immunohistochemistry was used to determine the tissue distribution of tight junction-related proteins and MUC5AC.

RESULTS. Transcripts for claudin-1, -2, -3, -4, -7, -9, and -14 were identified in human corneal epithelium. Transcripts for claudin-1, -2, -4, -7, -9, -10, and -14 were identified in human conjunctival epithelium. By immunohistochemistry, claudin-1, -4, and -7 were found to be localized at the membrane of human corneal and conjunctival epithelial cells. In human conjunctival epithelium, claudin-10 staining was observed at several, but not all, apical epithelial cell-to-goblet cell junctions.

CONCLUSIONS. Claudin-1, -4, and -7 are expressed in corneal and conjunctival epithelia. Claudin-10 is prominent at several junctions between apical epithelial cells and goblet cells in conjunctival epithelium. Except for claudin-10 expression in conjunctival epithelium, the claudin subtype expressions of corneal and conjunctival epithelia are similar. Therefore, there must be a difference between these two epithelial types with regard to the specific ratio of claudin subtypes expressed or their phosphorylation status. The distribution of goblet cells in conjunctival epithelium also influences the difference in barrier function. (Invest Ophthalmol Vis Sci. 2009;50:2103–2108) DOI:10.1167/iovs.08-3046

Corneal and conjunctival epithelium, which compose the ocular surface, form a barrier that isolates the eye from the outside environment and regulates the passive movement of fluid, electrolytes, macromolecules, and cells through the paracellular pathway. Tight junctions in the epithelium create this barrier, and together the cornea and conjunctiva work to form this important defense for the eye. However, it is well known that the tightness of the corneal epithelium tight junction is much greater than that of conjunctival epithelium.1,2 Corneal epithelium is composed of epithelial cells and has five or six layers of stratification. Conjunctival epithelium is also stratified squamous epithelium, but, unlike corneal epithelium, it contains goblet cells. Goblet cells are located in the apical surface of the conjunctiva and are interspersed among its multiple layers of stratified epithelium.

Tight junctions are present at the apical side of epithelia and play an important role in the establishment and maintenance of the barrier function and cell polarity. The tight junction is composed of three groups of proteins: transmembrane proteins (occludin, claudin, and junctional adhesion molecules); peripheral membrane proteins (ZO-1, ZO-2, ZO-3, MUPP-1), which have PDZ domains and bind to transmembrane proteins, and cytoplasmic proteins (cingulin, T6H antigen, etc.), which exist around tight junctions without any direct binding.3

Claudin (23 kDa) is composed of a family of transmembrane proteins that form the strands of the tight junction; 24 claudins have been identified thus far. Claudins are the only junctional proteins known to have tissue specificity. Occludin and claudins contain four transmembrane domains, with both N and C termini oriented into the cytoplasm, but these two proteins show no sequence similarity.4–6 Different mixtures of claudins and occludins create tight junction strands that are associated laterally with strands of adjacent cells, forming paired strands that eliminate extracellular space.7

We previously reported the distribution of ZO-1, occludin, and claudin in vivo human corneal epithelium.8 Immunohistochemistry has shown that in human corneal epithelium, most apical cells exhibit ZO-1, occludin, and claudin-1. With the use of reverse transcription-polymerase chain reaction (RT-PCR), the transcripts for claudin-1 and several other claudin isotypes (claudin-2, -3, -4, -7, -9, and -14) were identified from in vivo human corneal epithelium.

In this study, we examined the distribution of tight junction proteins ZO-1, occludin, and claudins from in vivo human conjunctival epithelium and sought to identify the difference between the tight junction properties of corneal and conjunctival epithelium.

MATERIALS AND METHODS

Tissue Preparation of Human Corneas and Conjunctivas

The experiments conducted in this study used human corneal tissue supplied by the Northwest Lion Eye Bank (Seattle, WA) or tissue extirpated from patients with corneal stromal opacity diseases during penetrative keratoplasty surgery (hence, the corneal epithelium was intact). Human conjunctival tissue was obtained at the time of cataract or conjunctival chalasis surgery with proper informed consent of the patients. All experiments were conducted immediately after the tissue was obtained. The present study had the approval of the Nantan General Hospital ethics committee, and the procedures followed the tenets of the Declaration of Helsinki.
Primary Antibodies

Rabbit anti–ZO-1 polyclonal antibody, rabbit anti–claudin-1 polyclonal antibody, rabbit anti–claudin-2 polyclonal antibody, rabbit anti–claudin-7 polyclonal antibody, rabbit anti–claudin-14 polyclonal antibody, and mouse anti–claudin-15 monoclonal antibody were purchased from Zymed Laboratories (South San Francisco, CA). Goat anti–occludin polyclonal antibody, goat anti–claudin-3 polyclonal antibody, goat anti–claudin-4 polyclonal antibody, goat anti–claudin-9 polyclonal antibody, and rabbit anti–claudin-10 polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti–MUC5AC monoclonal antibody was purchased from Abcam (Cambridge, UK). Alexa 488-labeled goat anti–rabbit antibody, Alexa 488-labeled rabbit anti–goat antibody, and Alexa 594-labeled goat anti–mouse antibody were purchased from Invitrogen Corporation (Carlsbad, CA).

Immunohistochemistry

Tight junction–associated proteins were studied by indirect immunohistochemistry. For transverse images, 7-µm cryostat sections were placed on gelatin-coated slides, air dried, and rehydrated in phosphate-buffered saline (PBS) containing 1.0 mM MgCl2 and 0.1 mM CaCl2 at room temperature for 15 minutes. Sections were fixed with 95% ethanol at 4°C for 30 minutes, followed by 100% acetone at room temperature for 1 minute. After several washes with PBS, sections were incubated with 1% bovine serum albumin at room temperature for 30 minutes to block nonspecific binding. Sections were then incubated at 4°C for 12 hours with primary antibody and were washed three times in PBS for 15 minutes. For negative controls, the equivalent serum was used. After staining with the primary antibodies, the sections were incubated at room temperature for 1 hour with suitable secondary antibodies and were washed several times with PBS.

For en face images, whole corneas were fixed with 95% ethanol at 4°C for 30 minutes, followed by 100% acetone at room temperature for 1 minute. After several washes with PBS, tissues were permeabilized by incubation in PBS containing 0.1% Triton X-100 for 10 minutes. Tissues were developed to the first and second antibody steps described. During all steps, the epithelial side was kept facing upward to avoid damage. Tissues were coverslipped with antifade mounting containing diamidino-2-phenylindole for nuclei staining (Vector Laboratories, Burlingame, CA). The slides were examined, and we took pictures every 0.5-µm slice and merged several pictures by confocal microscope (TCS SP2 AOBS; Leica Microsystems GmbH, Wetzlar, Germany).

RNA Isolation and RT-PCR Amplification of Claudin Species

For RT-PCR, human corneal epithelial sheets peeled from donor corneal buttons and excised conjunctiva were directly lysed with reagent (Trizol; Gibco BRL, Rockville, MD); total cellular RNA was isolated in accordance with the previous report by Yi et al.7 In brief, complementary DNA was generated in the presence of 0.5 µg oligo (dT) from 5 µg total RNA with reverse transcriptase (SuperScript II; Life Technologies, Rockville, MD). We used PCR primers designed by Yi et al.7 for human claudins-1 through -4, -7, -9, -10, -14, and -15. PCR products were examined by 2% agarose gel and ethidium bromide staining. The observed PCR products corresponded to their expected molecular weights.

RESULTS

Distribution of ZO-1 and Occludin in Human Conjunctival Epithelium

In the transverse sections, occludin and ZO-1 were localized at the apical superficial epithelial cell tight junctions and the epithelial cell to goblet cell tight junctions (Figs. 1A, C). In the en face sections, occludin and ZO-1 antibodies showed as bands that corresponded to the junctional complex (Figs. 1B, D). This occludin staining was different from that of the cornea, as it was not continuous and was presented in a dotlike pattern along the cell junctions.8

Claudin Subtype Expression in Human Conjunctival Epithelial Cells Detected by RT-PCR

We previously reported that the transcripts for claudin-1 and several other claudin isotypes, such as claudin-2, -3, -4, -7, -9, and -14, were identified from human corneal epithelium.8 In this study, we determined the presence of transcripts of claudin subtypes in human conjunctival epithelial cells by the same RT-PCR method. The transcripts for claudin-1, -2, -3, -4, -7, -9, -10, and -14 were identified from conjunctival epithelium (Fig. 2). Claudin-3 was not identified in human conjunctival epithelium but was present in human corneal epithelium. On the other hand, claudin-10 was identified in conjunctival epithelium but not in corneal epithelium. No amplified claudin mRNA was observed without reverse transcriptase (data not shown).

Distribution of Claudins in Human Conjunctival and Corneal Epithelium

Not all claudin subtypes whose transcripts were identified were expressed in conjunctival and corneal epithelia. The corneal epithelial cells through all cell layers were stained by claudin-1,-4,-7,-9,-10,-14,-15 was observed (Fig. 3). In the en face images, claudin-1,-4,-7,-9,-10,-14,-15 was observed in all cell layers. Claudin-7 staining was observed in superficial cells (Fig. 5). In the en face images, those three claudin subtype antibodies showed as bands that corresponded to the junctional complex (Fig. 6). In addition, some openings of goblet cells that were identified by positive reactivity with anti–MUC5 antibody showed claudin-10 staining (Figs. 5, 6). No staining was observed by claudin-2,-3,-9,-14,-15 (Fig. 6).
DISCUSSION

The ocular surface consists of corneal and conjunctival epithelia, both of which are stratified nonkeratinized epithelium. The corneal epithelium is a transparent, flat, stratified squamous epithelium devoid of goblet cells. It has a cuboidal basal layer lying on the avascular corneal stroma by the Bowman layer. The conjunctival epithelium is populated by goblet cells. Sequencing of mucin genes has led to the identification of two categories of mucins, secreted and membrane associated. Conjunctival goblet cells express one of the secreted gel-forming mucins, MUC5AC.10 Like epidermis and other surface-lining mucosa, corneal and conjunctival epithelia serve as barriers of the ocular surface. This barrier is crucial for maintaining the homeostasis of fluid and solutes between the intraocular milieu and precorneal tear film. Although corneal and conjunctival epithelia provide barrier functions at the ocular surface together, the barrier function of corneal epithelium is much stronger than that of conjunctival epithelium.1,2

There are many differences between corneal and conjunctival proteome. For example, it is widely known that differentiated human corneal epithelial cells express cytokeratin 3 and cytokeratin 12. In addition, other cytokeratins, including cytokeratin 14 and cytokeratin 19, are expressed as minor components of the cytoskeleton in basal and suprabasal human corneal epithelial cells. On the other hand, conjunctival epithelium uniformly expresses cytokeratin 19 but not cytokeratin 12.11 The distribution of α-subchains of type IV collagen in the basement membrane is also different between corneal and conjunctival epithelia. The conjunctival basement membrane contains collagen α2(IV) but not

![Claudin subtype expression in human corneal epithelium in the transverse sections by immunofluorescence staining. Corneal epithelial cells through all cell layers were stained by claudin-1, -4, and -7. No staining was observed by claudin-2, -3, -9, -10, -14, or -15. Blue: nuclear counterstaining. Scale bar, 50 μm.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932959/)

![Claudin subtypes](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932959/)

Lane M: molecular weight marker. Scale bar, 500 bp.
collagen α5(IV). By contrast, collagen α5(IV) is found in the corneal basement membrane, but collagen α2(IV) is not.\textsuperscript{12}

Tight junctions are present at the apical side of epithelium and play an important role in the establishment and maintenance of barrier function and cell polarity. Barrier characteristics of tight junctions vary considerably among different types of epithelium and endothelium, depending on physiological requirements.\textsuperscript{13}

Occludin (60 kDa) was the first transmembrane protein identified at tight junctions,\textsuperscript{14} but its precise cellular functions remain unclear. Occludin-deficient mice are viable; the tight junction ultrastructure appears unaltered, and isolated intestinal tissues demonstrate normal transepithelial resistance (TER) and permeability to mannitol.\textsuperscript{15,16} However, blocking the extracellular loops\textsuperscript{17} and reducing the protein content of occludin\textsuperscript{18} alter paracellular permeability in a number of cell systems. The function of occludin in regulating epithelial cell division has been suggested by the ability of exogenous occludin expression to revert the phenotype of Raf-transformed rat salivary gland epithelial cells.\textsuperscript{19}

Claudin (23 kDa) is composed of a family of transmembrane proteins that form the strands of the tight junction.\textsuperscript{4} Occludin and claudins each contain four transmembrane domains, with both N and C termini oriented into the cytoplasm, but these two proteins show no sequence similarity. Twenty-four claudins have been identified thus far. Sequence analysis of claudins has led to differentiation into two groups, designated as classic claudins (claudins 1–10, 14, 15, 17, 19) and nonclassic claudins (claudins 11–13, 16, 18, 20–24), according to their degree of sequence similarity.\textsuperscript{20}

Claudins are the only junctional proteins known to have tissue specificity. Different mixtures of claudins create tight junction strands that are associated laterally with strands of adjacent cells, thus forming paired strands that eliminate extracellular space. However, it has been postulated that ion-selective pores occur within paired tight junction strands.\textsuperscript{5,7,21}

All claudins have two extracellular loops. The first extracellular loop consists of approximately 50 amino acids with two conserved cysteines. The distribution of the charged amino acid residues in the first extracellular loop of claudins is crucial for determining the charge selectivity of the aqueous pores of tight junction strands.\textsuperscript{22} The second extracellular loop usually has approximately 25 amino acids. It may associate with itself and may have a holding function, narrowing the paracellular cleft.\textsuperscript{23}

Claudin-13 has no human expressed sequence tags (ESTs), and most murine ESTs for claudin-13 are from embryonic DNA libraries, thus suggesting that these genes may not be expressed in adult tissues. Claudin-6 is developmentally restricted and is not expressed in adult tissues.\textsuperscript{24} Claudin-11 has been found only in oligodendrocytes and Sertoli cells in the testis.\textsuperscript{25} Morita et al.\textsuperscript{26} report that claudin-5/TMVCF is only expressed in the endothelial cells of blood vessels. Claudin-16/parecel-lin-1 is expressed exclusively in the thick ascending limb of Henle and may form aqueous pores that function as Mg\textsuperscript{2+}...
paracellular channels. We eliminated those subtypes from our experiment.

Human corneal epithelial cells through all cell layers were stained by claudin-1,-4, and -7. No staining was observed by claudin-2,-3,-9,-10,-14, and -15. In the en face images, claudin-1,-4, and -7 antibodies showed as bands that corresponded to the junctional complex. In the human conjunctival epithelium, claudin-1 and -4 staining was observed in all cell layers. Claudin-7 staining was observed in superficial cells. In the en face images, those three claudin subtype antibodies showed as bands that corresponded to the junctional complex. In addition, some openings of goblet cells showed claudin-10 staining. In the present study, we investigated the mRNA expression of claudins by RT-PCR and localization by immunofluorescence microscopy. We observed discrepancies between mRNA and the protein expressions of claudins. Transcripts for claudin-1,-2,-3,-4,-7,-9, and -14 were identified in human corneal epithelium. Transcripts for claudin-1,-2,-4,-7,-9,-10, and -14 were identified in human conjunctival epithelium. There are several possibilities for these discrepancies, including low levels of the translation of claudin mRNAs into proteins, rapid protein turnover, and low amounts of claudin proteins in tissue.

Claudin-1 is ubiquitous. In mammalian skin, continuous tight junctions circumscribing the keratinocytes of the granular cell layer were reproducibly identified, and claudin-1 and -4 were concentrated in these tight junctions. Claudin-1-deficient mice were born alive but died within 1 day of birth accompanied by excessive water loss from the skin. Claudin-10 expression has been reported in the inner ear, mouse prostate, most segments of nephron, endothelial cells of restricted blood vessels, colon epithelium, and exocrine glands. In exocrine glands, including the submandibular, sublingual, parotid, and lacrimal glands, claudin-10 was expressed along lateral membranes in addition to apical tight junction strands.

As discussed, claudins are transmembrane proteins that form tight junction. Electron microscope freeze-fracture observation and horseradish peroxidase permeability study revealed that the tight junction exists only between superficial epithelial cells in corneal epithelial cells. However, our study demonstrated the existence of claudins at all cell layers. Several reports describe claudin proteins as expressed not only at tight junctions but also along the lateral membrane. For example, claudin-1,-4, and -7 were localized along the lateral membrane in the airway epithelium. Although the biological significance of the localization of claudin proteins in the lateral membrane is unknown, we speculate that because the surfaces of corneal and conjunctival epithelia are always exfoliating and because the turnover of the epithelium is 7 to 10 days, claudin proteins might exist at the membrane that allows rapid formation of tight junction strands.

In conclusion, the results of our study showed that claudin-1,-4, and -7 were expressed in corneal and conjunctival epithelia. We also found that claudin-10 was prominent at several junctions between apical epithelial cells and goblet cells in conjunctival epithelium. Because variations in the tightness of individual paired tight junction strands are determined by the combination of claudin species and because the barrier function of corneal epithelium is stronger than that of conjunctival epithelium, we speculated at the beginning of this experiment that subtype expression in these two types of epithelia might be different. Except for claudin-10 expression in conjunctival epithelium, however, claudin subtype expression of corneal and conjunctival epithelia is similar. Therefore, we posit that there must be a difference between these two types of epithelium regarding the specific ratio of claudin subtypes expressed or their phosphorylation status and that the distribution of goblet cells in conjunctival epithelium and claudin-10 expression between epithelial cells and goblet cells also influences the difference in barrier function between these two types of epithelium. The elucidation of claudin subtype expression in specific tissue is an important first step in developing a...
strategy to regulate drug absorption or to prevent some diseases. In this study, we demonstrated the claudin subtype expression in corneal and conjunctival epithelia. Further investigations are required into the regulation of the pores made by those claudins.

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References
