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 both 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/or suprabasal human corneal epithelial cells. On the other hand, conjunctival epithelium uniformly expresses cytokeratin 19, but not cytokeratin 12.[11] The distribution of alpha sub-chains of type IV collagen in the basement membrane is also different between corneal and conjunctival epithelium. The conjunctival basement membrane contains collagen alpha2(IV), but not collagen alpha5(IV). By contrast, in the corneal basement membrane, there is the collagen alpha5(IV), but not the collagen alpha2(IV). [12]

Tight junctions are present at the apical side of epithelia and play an important role in the establishment and maintenance of barrier function and cell polarity. The barrier characteristics of tight junctions vary considerably among different types of epithelium and endothelium depending on physiological requirements.[13]

Occludin (60kDa) was the first transmembrane protein identified at tight junctions,[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 trans epithelial resistance (TER) and permeability to mannitol.[15, 16] However, blocking the

extracellular loops[17] and reducing the protein content of occludin[18] alter paracellular permeability in a number of cell systems. On the other hand, 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.[19]

Claudin (23 KDa) is comprised of a family of transmembrane proteins that form the strands of the tight junction.[4] Both 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. 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 non-classic claudins (claudins 11-13, 16, 18, 20-24), according to their degree of sequence similarity.[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.[5, 7, 21] All claudins have two extracellular loops. The first extracellular loop consists of ~ 50 amino acids with two conserved cysteines and 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.[22] The second extracellular loop usually has ~ 25 amino acids and may associate with itself and possess a holding function, narrowing the paracellular cleft.[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 not expressed in adult tissues.[24]

Claudin-11 has been found only in oligodendrocytes and Sertoli cells in the testis.[25] Morita et al. reported that claudin-5/TMVCF is only expressed in the endothelial cells of blood vessels.[26] Claudin-16/parecellin-1 is exclusively expressed in the thick ascending limb of Henle and might form aqueous pores that function as Mg^{++} paracellular channels.[27] We eliminated those subtypes from our experiment.

The 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 were 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, it was worth mentioning that 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. There were discrepancies between mRNA and the protein expressions of claudins. The transcripts for claudin-1, -2, -3, -4, -7, -9, and -14 were identified from human corneal epithelium. The transcripts for claudin-1, -2, -4, -7, -9, -10, and -14 were identified from human conjunctival epithelium. There are several possibilities for these discrepancies including low levels of the translation of claudin mRNAs into proteins, rapid protein turnover, or low amounts of claudin proteins in tissues.

Claudin-1 is ubiquitous and common. 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 one day of birth accompanied by excessive water loss from the skin.[28]

Clostridium perfringens enterotoxin (CPE) is a single polypeptide and can cause food poisoning in humans. Katahira et al. identified the receptor on the cell membrane for CPE (CPE-R).[29] Because of the significant sequence similarity for claudin-1 and -2, Morita et al. found that CPE-R was identical to claudin-4. [30] The treatment of the cells with C-CPE reduces the TER. [31] Claudin-4 has a tightening potential. Claudin-4 increased TER ~300% when expressed in low-resistance Madin-Darby canine kidney (MDCK) II cells and decreased the paracellular permeability for Na^+ more than Cl^-.[32] On the other hand, paracellular cation pores are formed by claudin-7 for Na^+. The mouth of the channel, which is constituted by extracellular domains of claudin-7 from opposing cells, is negatively charged and hinders Cl^- entry while allowing Na^+ to go through.[33]

Claudin-10 expression in the inner ear, mouse prostate, most segments of nephron, endothelial cells of restricted blood vessels, colon epithelium, and exocrine glands has been reported.[34-37] 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.[37]

As mentioned above, claudins are tight junction forming transmembrane proteins. Electron-microscopic freeze-fracture observation and horseradish peroxidase permeability study revealed that the tight junction exists only between superficial epithelial cells in corneal epithelial cells.[38,39] However, our study demonstrated the existence of claudins at all cell layers. There are several reports describing claudin proteins as being 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.[40] Although the biological significance of the localization of claudin proteins in the lateral membrane is unknown, we speculate that since the surface of corneal and conjunctival epithelium is always exfoliating and since 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 both 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. Since variations in the tightness of individual paired tight junction strands are determined by the combination of claudin species, and since the barrier function of corneal epithelium is much stronger than that of conjunctival epithelium, we speculated at the beginning of this experiment that the subtype expression in these two types of epithelium might be different. However, and except for the claudin-10 expression in conjunctival epithelium, the claudin subtype expression of corneal and conjunctival epithelium is similar. Therefore, we posit that there must be a difference between these two types of epithelium in regards to the specific ratio of claudin subtypes that are 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 influence the difference in barrier function that exists between these two types of epithelium. The elucidation of claudin subtype expression in specific tissue is an important first step for developing a strategy for regulating drug absorption or for preventing some diseases. In this report, we demonstrated the claudin subtype expression in corneal and conjunctival epithelium. Further investigations into the regulation of the pores which are made by those claudins will be required.

Acknowledgments

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Figure legends

Figure 1. Distribution of ZO-1 and occludin in human conjunctival epithelium by immunofluorescence staining. In the transverse sections, ZO-1 (A) and occludin (C) were localized at the apical superficial epithelial-cell tight junctions, as well as the epithelial-cell to goblet-cell tight junctions. In the en face images, ZO-1 (B) and occludin (D) antibodies showed as bands that corresponded to the junctional complex. Red: goblet cells labeled by mucin staining with anti-MUC5AC antibody. Blue: nuclear counterstaining. Scale bar: 50μm.

Figure 2. Claudins expressed in human corneal and conjunctival epithelial cells detected by RT-PCR. The transcripts for claudin-1, -2, -3, -4, -7, -9 and -14 were identified from human corneal epithelium. The transcripts for claudin-1, -2, -4, -7, -9, -10, and -14 were identified from conjunctival epithelium. M lane: molecular weight marker, Bar: 500 bp.

Figure 3. Claudin subtype expression in human corneal epithelium in the transverse sections by immunofluorescence staining. The 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. Blue: nuclear counterstaining. Scale bar: 50μm.

Figure 4. Distribution of Claudin-1, -4, and -7 in human corneal epithelium in the en face images by immunofluorescence staining. Claudin-1, -4, and -7 antibodies showed as bands that corresponded to the junctional complex. Blue: nuclear counterstaining. Scale bar: 50μm.

Figure 5. Claudin subtype expression in human conjunctival epithelium in the transverse sections by immunofluorescence staining. Claudin-1 and -4 staining in all cell layers of human conjunctival epithelium were observed. Claudin-7 staining was observed in superficial cells. Some openings of goblet cells showed claudin-10 staining (arrow). Red: goblet cells labeled by mucin staining with anti-MUC5AC antibody. Blue: nuclear counterstaining. Scale bar: 50μm.

Figure 6. Claudin subtype expression in human conjunctival epithelium in the en face images by immunofluorescnece staining. Claudin-1, -4, and -7 antibodies showed as bands that corresponded to the junctional complex. In addition, some openings of goblet cells showed claudin-10 staining (arrow). No staining was observed by claudin-2, -3, -9, -14, and -15. Red: goblet cells labeled by mucin staining with anti-MUC5AC antibody. Blue: nuclear counterstaining. Scale bar: 50μm.

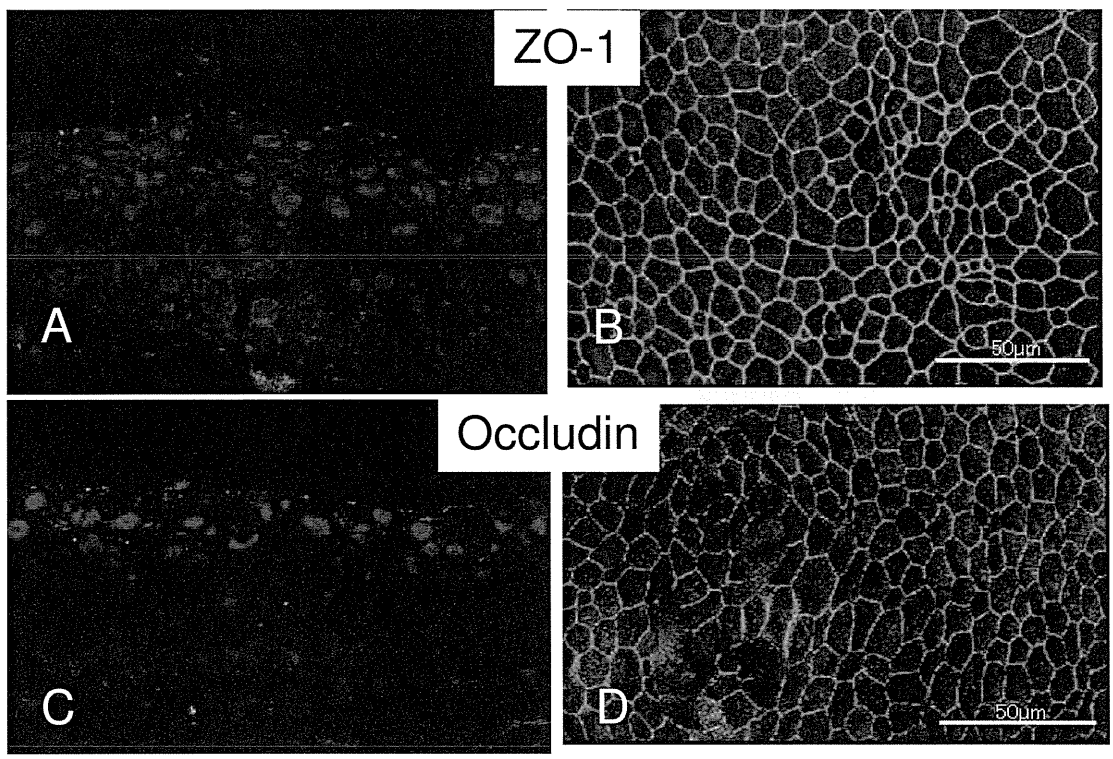

Figure 1

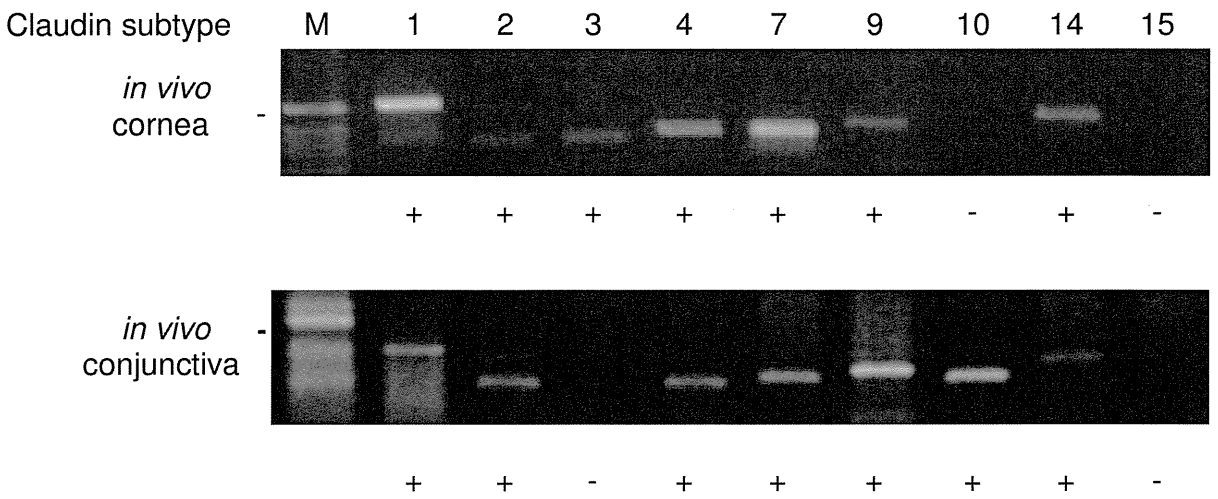
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Figure 2

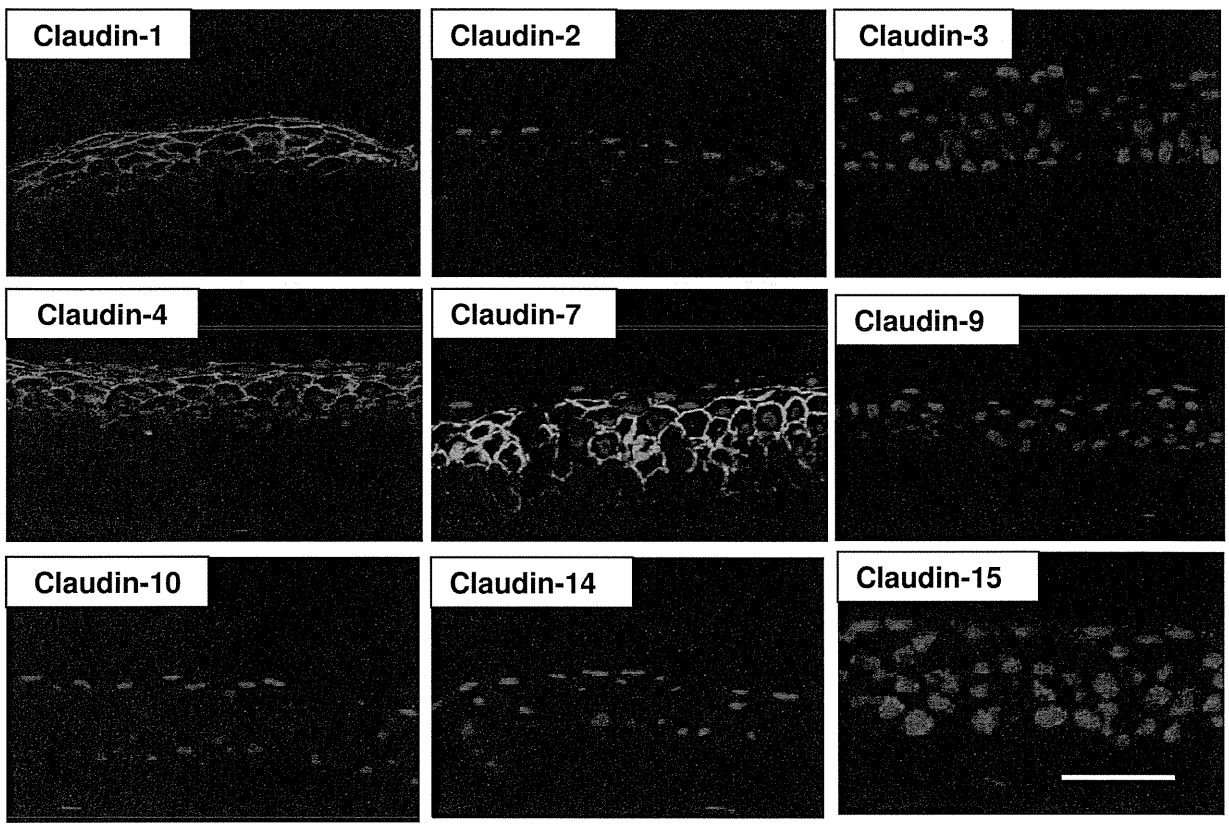
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Figure 3

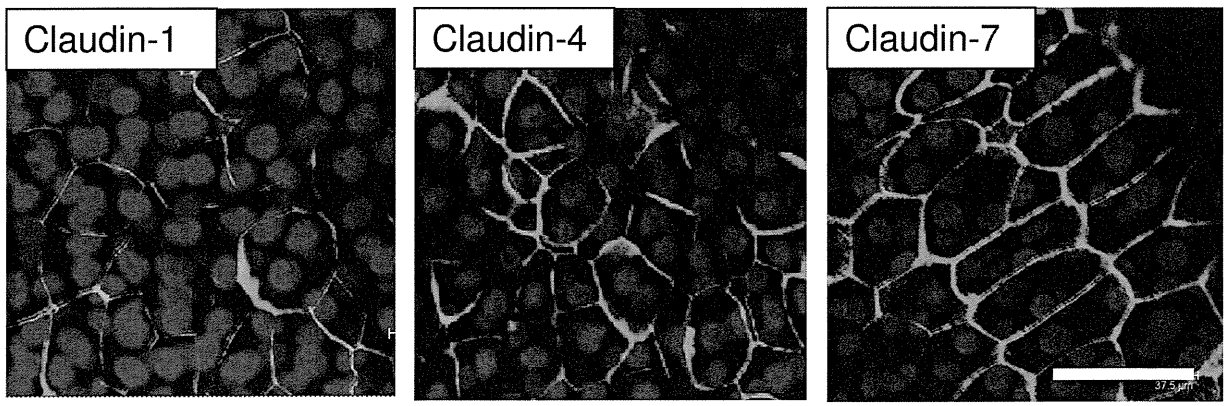

Figure 4

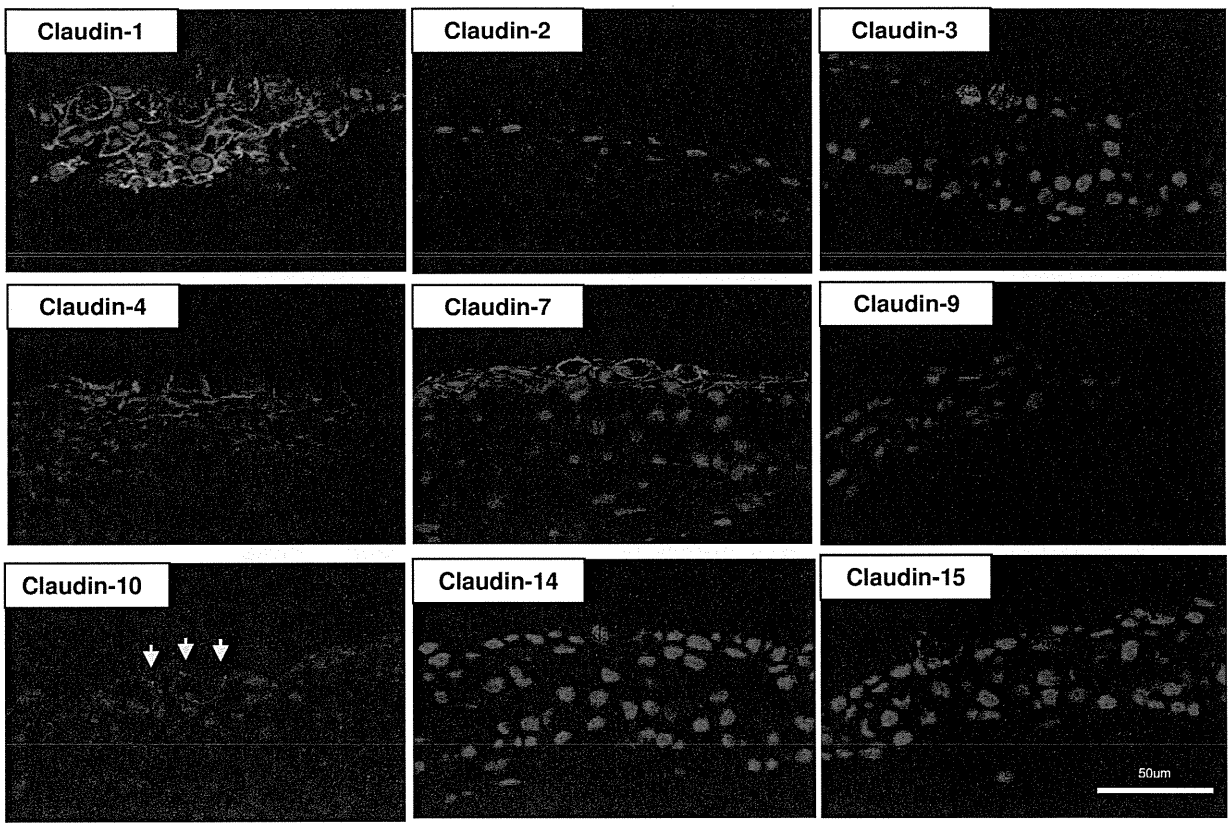
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Figure 5

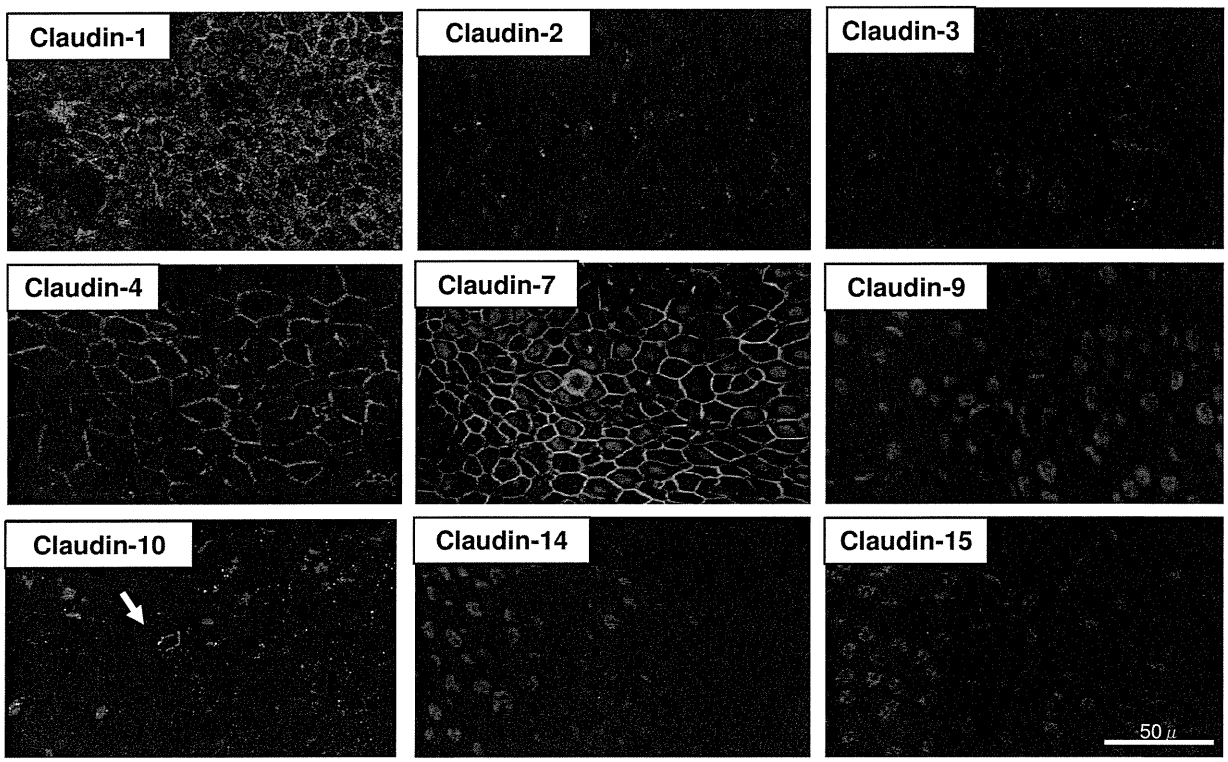
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Figure 6

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Applying Magnetic Bead Separation / MALDI-TOF Mass Spectrometry to Human Tear Fluid Proteome Analysis

Eiichi Sekiyama[1], Yumiko Matsuyama[2], Daisuke Higo[2], Takashi Nirasawa[2], Masaya Ikegawa[3*], Shigeru Kinoshita[1], and Kei Tashiro[3]

[1]Kyoto Prefectural University of Medicine, Ophthalmology
[2] Bruker Daltonics
[3]Kyoto Prefectural University of Medicine, Genomic Medical Sciences

*Corresponding author: Masaya Ikegawa, Genomic Medical Sciences, Kyoto Prefectural University of Medicine, 465 Kawaramachi Hirokoji, Kamigyo-ku, Kyoto, Japan
Postal code: 602-8566; Tel & Fax: +81-75-251-5347; E-mail: mikegawa@koto.kpu-m.ac.jp

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Key words: tear fluid; magnetic bead; Proline-rich protein 4; ClinProt; MALDI-TOF-MS

Abstract

The proteins and peptides in tears play an important role in preserving the integrity and stability of the ocular surface. Proteomic analysis of tear films will enable us to detect early biological markers of eye diseases, however, it is often hampered by the small amount of tear volume and the low protein concentration. Here we adopted magnetic bead-based purification (ClinProt system) followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to profile human tear proteins. Basal and reflex tear fluids were collected from normal healthy volunteers using glass microcapillary tubes. Reversed phase (C8) and weak cation exchange (WCX) magnetic beads were applied to obtain multiple components detected as clear signals. Principal component analysis showed a clear differentiation between basal and reflex tears. Among the key alterations, two markedly increased peaks in the reflex tear fluids at m/z 2422.12 and m/z 2721.29 were subsequently analyzed by tandem MS analysis and their source to be proline-rich protein 4 (PRP4). We conclude that magnetic bead-based separation combined with MALDI-TOF-MS (ClinProt MALDI-TOF) appears to be ideally suited for the first-line screening of peptides and proteins in tears.

The search for biomarkers of human diseases has been increasingly successful because of emerging new techniques in the field of proteomics (Hu, 2006; Villanueva, 2004; Zhang, 2004; Ketterlinus, 2005; Koo, 2005; Cheng AJ, 2005; Mirr EN, 2005). Proteins and peptides in tears are reported to play important roles in preserving the integrity and stability of the ocular surface, and changes in tear proteins eins are associated with various pathological eye conditions (Koo, 2005). Among the molecules identified are candidates for biomarkers of dry-eye diseases (Grus, 2005; Tomosugi N, 2005). Earlier investigations of tear film proteins have included extensive analysis using high-performance liquid chromatography (HPLC) or two-dimensional (2-D) gel electrophoresis, combined with mass spectrometry-based protein identification (Koo, 2005; Cheng, 2005; Mirr, 2005; Grus, 2005; Tomosugi, 2005; Kijlstra 1989; Zhou, 2006; Li, 2005; Fung, 2004; de Souza, 2006), but these protocols are sometimes hampered by the small amount of tear fluid and its low protein concentration. For high-throughput analysis, surface-enhanced laser desorption / ionization time-of-flight (SELDI-TOF) MS analysis was developed (Grus, 2005; Tomosugi N, 2005). With this technique, very small sample volumes can be directly applied to chip-based array surfaces; however, its limitations include the difficulty of further protein identification. Here we show that the combination of magnetic bead separation and MALDI-TOF MS spectrometry (ClinProt system) is a reasonably efficacious, simple method for profiling and identifying proteins from eluted tear fluids.

Open-eye basal tear fluids were collected from twenty normal healthy volunteers who did not wear contact lenses and had no evidence of ocular disease. The subjects ranged in age from 20 to 29 years, old enough to collect properly physiological tears as described below. Informed consent was obtained from all volunteers participating in the study, and the protocols were approved by the institutional ethics committee and conformed to the provisions of the Declaration of Helsinki. The ophthalmic examination included subjective symptoms, Schirmer's test, biomicroscopy with careful examination of the lid margin and meibomian glands, and tear break-up time. Each volunteer was questioned about subjective symptoms such as burning, itching, foreign body sensation, dryness, and photophobia. Tear fluid was collected in the afternoon using 1-μL glass micro-capillary tubes (Corning, New York, NY, USA) without touching the lid margins or eye-lashes. After basal tear fluids were collected, reflex tear fluids were elicited by nasopharyngeal scrub and collected. The collected samples were stored at -80 °C until analysis.

For analysis, the tear fluid samples were thawed and purified with a reagent set that included two kinds of chemically coated magnetic beads: reversed phase (C8) and weak cation exchange (WCX) (ClinProt™ Bruker Daltonics). We used œ-cyano-4-hydroxycinnamic acid as the matrix solution. All these procedures were performed at room temperature with moderate humidity. The eluted samples were then dropped onto a MALDI sample plate (600 μm Anchorchip™: Bruker Daltonics), and spectra were obtained by an Autoflex II MALDI-TOF mass ss spectrometer (Bruker Daltonics) operated in positive-ion linear mode. All spectra were obtained randomly over the surface of the matrix spot. The criteria for peak detection were: signal-to-noise ratio >5 and 2-Da peak-width filter. Approximately 10-20 peaks were produced after the treatment with the WCX or C8 beads (Fig. 1A). Multiple components were detected as clear signals in the mass range of 0-20 kDa, which includes proteins such as lysozyme and lipocalin. Inducible secreted tear proteins are believed to consist primarily of three entities that account for 85% of the total protein content: lysozyme, lactoferrin, and the tear-specific lipocalins (Kijlstra, 1989). Lysozyme and lipocalin were previously identified as protein fragments at *m/z* 14,687.8 and *m/z* 17,438, respectively (Fung, 15 2004). In the present study, we detected signals with the same *m/z* ratios in the basal tear fluid samples (Fig.1A). However, we could not detect lactoferrin by MALDI-TOF MS analysis with WCX or C8 beads, or by electrospray ionization (ESI)-MS analysis, for unknown reasons (Kijlstra, 1989).

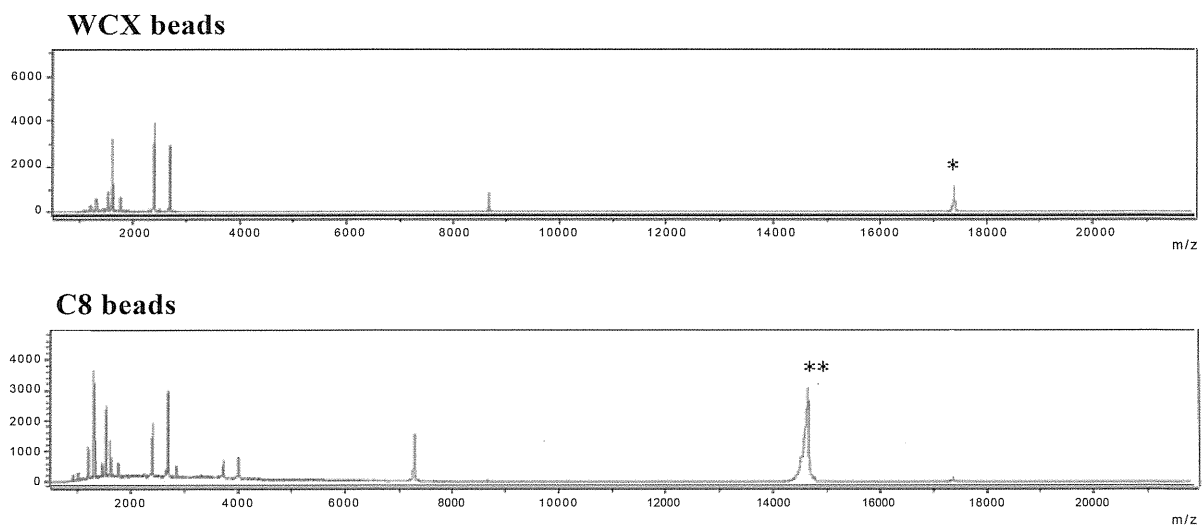

Figure 1: Protein/Peptide profiling of tear fluid samples from twenty healthy volunteers using ClinProt Mass Spectrometry.

(A) Typical ClinProt profiles of basal tear fluids eluted from WCX and C8 beads in the mass range 0-20 kDa m/z and subjected to flexAnalysis™. Multiple components were detected as clear signals, including lipocalin (*: m/z 17438) and lysozyme (**: m/z 14687.8).

The obtained data were graphed as columns representing normalized peak intensities (Fig.1B; pseudo-gel view) and further analyzed by a multivariate statistical analysis including principal component analysis (PCA) by the *ClinProTools*TM software (Bruker Daltonik) (Zhang, 2004; Ketterlinus, 2005). The results showed a differential distribution of samples from basal tears ars from reflex tears (Fig.1C).

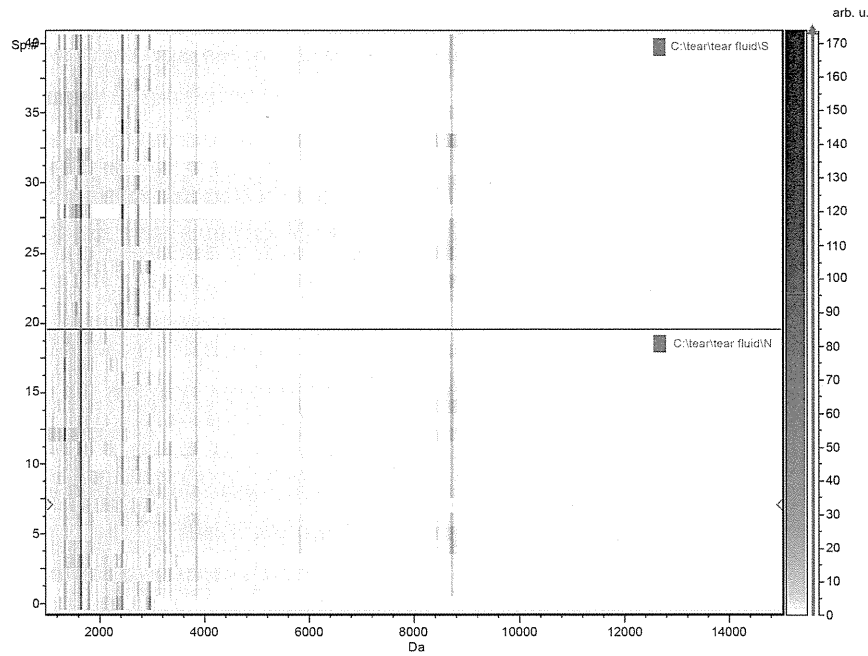

Figure 1: (B) Psuedo-gel views of the mass spectrum of basal tear fluids (lower column) and the reflex tear fluids (upper column) were shown with the calculated molecular weight (m/z values) along the x-axis and relative intensity along the y-axis using ClinProtools™.

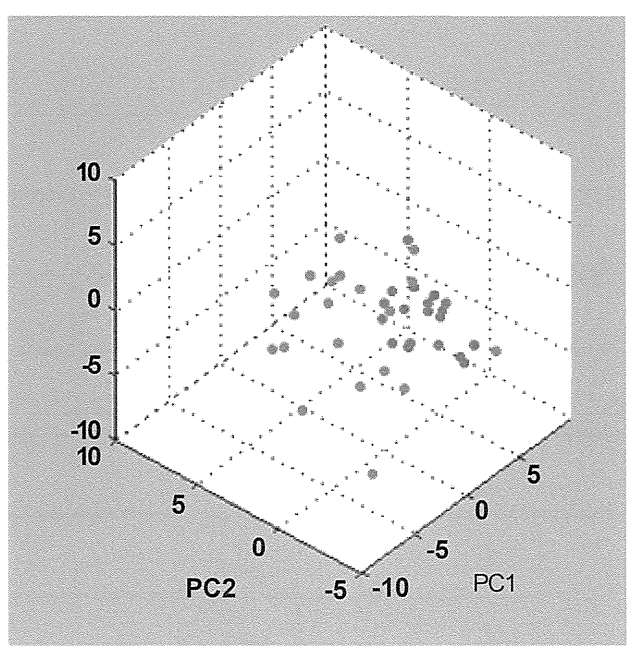

Figure 1: (C) 3-D view of PCA scores plot analyzed by *ClinProtools*™. Green spots represent reflex tears and the red spots represent basal tears.