Leakage of Aquaporin 5 in the Tear of Dacryoadenitis Mice

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PURPOSE. The objective of this study was to investigate whether leakage of aquaporin 5 (AQP5) in tear is associated with damage of lacrimal glands (LGs) in dacryoadenitis models.

METHODS. Female MRL/lpr (24-week-old), male NOD/Shi Jci (5-, 8-, and 10-week-old), female NFS/s-TX (10-week-old), and lipopolysaccharide (LPS)-induced dacryoadenitis model mice were used. Tear fluid was collected by a cotton thread. Tear proteins in the thread were dissolved in sodium dodecyl sulfate buffer, and AQP5 proteins were analyzed by the Western blot technique using anti-AQP5 antibody. LGs were prepared for hematoxylin and eosin staining or immunostaining of AQP5.

RESULTS. In MRL/lpr, NFS/s-TX, 8- and 10-week-old NOD/Shi Jci mice, AQP5 protein was detected in the tear by Western blot analysis. Inflammatory lymphocyte infiltrations were observed in LGs of these dacryoadenitis model mice. In contrast, AQP5 leakage and damage of LG were not observed in normal mice. In 5-week-old NOD/Shi Jci mice, infiltration was not seen in LG, and AQP5 leakage was not detected in the tear. In LPS-induced dacryoadenitis model mice, either tissue destruction with inflammation in LG or AQP5 leakage in the tear was observed. AQP5 in the tear and tissue inflammation in LGs was not found in control mice. These results indicate that AQP5 is leaked in tears when LGs are damaged by dacryoadenitis.

CONCLUSIONS. Leakage of AQP5 in the tear was found to be related to LG damage. This finding suggests that detection of AQP5 in tear is useful for specific diagnosis of LG disorders with tissue destruction. (Invest Ophthalmol Vis Sci. 2000;41:2432–2437)

In chronic dacryoadenitis, Sjögren’s syndrome is a systemic autoimmune disorder characterized by dry eye and dry mouth. This dryness results from lymphocyte infiltration into lacrimal and salivary glands.1 In acute dacryoadenitis, intralobular edema, lymphocytes, and plasma cell infiltration were observed in the lacrimal gland.2 In Sjögren’s syndrome, it is assumed that infiltrated T cells may recognize unknown self-antigens and produce inflammatory cytokines and autoantibodies.3 Histopathologically, lymphocytes penetrate the epithelium of the salivary gland ducts and cause cytolysis of lacrimal gland cells.4 To diagnose these kinds of damage to the lacrimal gland, an histologic evaluation of biopsy specimens is widely used. However, this procedure has limitations. The biopsy is not available for reexamination and causes discomfort to the patients. The evaluation of reflex tearing is also used for diagnosis of Sicca syndrome. It has been reported that poor reflex tearing is associated with lymphocyte infiltrations in the lacrimal gland.4 Computed tomography or magnetic resonance imaging of the glands is another procedure used for the diagnosis of lacrimal tumors.5 However, these methods are not always specific or convenient for diagnosis. Hence, a potential technique that can obviate biopsy, or specific markers to detect lacrimal gland damage, is needed.

Recently, functional water channels have been identified in mammals, and this group of proteins has been referred as the aquaporins.6 We have shown that aquaporin 5 (AQP5) protein is specifically localized in the apical membrane of acinar and duct cells in mouse7 and human (Tsubota K, Hirai S, et al., unpublished data, 2000) lacrimal gland. In other ocular tissues, AQP5 expression was not demonstrated in conjunctiva.8 In contrast to AQP5, membrane channel proteins, such as ion channels or sodium/potassium adenosinetriphosphatase, have been shown as present not only in the lacrimal gland9,10 but also in the corneal epithelium11 and the conjunctiva.12 To the best of our knowledge, no specifically localized protein in the lacrimal gland other than AQP5 has been reported. Based on this evidence, we hypothesize that AQP5 is specifically leaked into tear fluid when the lacrimal gland is damaged by lymphocyte infiltrations and inflammation with tissue destruction. In central diabetes insipidus,13 impaired water excretion, and hyponatremia,14 aquaporin 2 (AQP2), which was excreted into urine, was found to be related to the pathologic state of the disease. In this study, we demonstrate that in experimental dacryoadenitis models, the detection of AQP5 in tear fluid is useful for specifically diagnosing lacrimal gland damage.

METHODS

All experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Dacryoadenitis models were female MRL/lpr, male...
NOD/Shi Jci (Japan CLEA, Tokyo, Japan), female NFS/s-TX, and lipopolysaccharide (LPS)-induced dacryoadenitis mice. These mice were maintained in a specific pathogen-free colony and given food and water ad libitum. NFS/s/TX mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). They were thymectomized on day 3 after birth. Thymectomized NFS/s/TX mice are referred to as NFS/s-TX mice. As control animals, we purchased female BALB/c for MRL/lpr, male Jc1:ICR for NOD/Shi Jci, and female NFS/s-nonTX (non-thymectomized mice) for NFS/s-TX mice, respectively. MRL/lpr and BALB/c were killed at 24 and 7 weeks after birth, respectively. NFS/s-TX and NFS/s-nonTX mice were killed at 10 weeks. NOD/Shi Jci and Jc1:ICR mice were killed at 5, 8, and 10 weeks. As LPS-induced dacryoadenitis model mice, 5-week-old male BALB/c mice were anesthetized with sodium pentobarbital (60 mg/kg), and injected with saline-dissolved LPS (25 μg/μl; Difco, Detroit, MI) into the exorbital lacrimal gland. Saline-injected mice were used as control animals. Mice in this group were killed at 3, 9, and 24 hours after injection.

For immunoblot detection of AQP5 in the tear fluid, mice were anesthetized with sodium pentobarbital (60 mg/kg), and the tears were collected with a cotton thread treated with the phenol red pH indicator (Showa Yakuhin Kako, Tokyo, Japan) used in Schirmer’s test. Tear volume was determined by the length of wet thread indicated with phenol red pH indicator. The unit of length was the millimeter. Tear proteins in 10 mm length of wet thread indicated with phenol red pH indicator were dissolved in 10 mM Tris–HCl, pH 7.4, 4% sodium dodecyl sulfate, 20% glycerol, 5% 2-mercaptoethanol) and electrophoresed on a 10% to 20% gradient polyacrylamide gel. Tear proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) and immunolabeled using affinity-purified anti-AQP5 IgG at a 1:1000 dilution. Anti-AQP5 rabbit IgG that recognizes the carboxyl-terminus of rat AQP5 was prepared as previously described. The bound antibodies were detected using the ECL chemiluminescence method (Amersham, Buckinghamshire, UK). For positive control of immunoblot detection of AQP5, a membrane fraction was isolated from BALB/c mouse lacrimal glands. Glands were homogenized in a buffer solution of 30 mM Tris–HCl, pH 7.2. After fixation, lacrimal glands were dehydrated with ethanol and embedded in paraffin. Tissue sections (4-μm-thick) were prepared for hematoxylin and eosin (HE) staining or immunohistochemical staining of AQP5. Immunohistochemistry was performed on lacrimal gland sections of NOD/Shi Jci and Jc1:ICR mice using anti-AQP5 guinea pig IgG that recognizes the extracellular domain of rat AQP5. Sections were stained with phosphate-buffered saline (PBS) at pH 7.2 and incubated with 0.1% trypsin in PBS for 30 minutes. After incubation, sections were rinsed with PBS and treated with methanol containing 0.3% H2O2 for 15 minutes. Sections were rinsed and incubated with blocking agent (15 μl/ml normal goat serum, 1% bovine serum albumin, 0.5% Triton-X in PBS, pH 7.2) for 1 hour at room temperature. After incubation with anti-AQP5 antibody at a 1:1000 dilution overnight at 4°C, sections were rinsed with PBS 3 times for 5 minutes and treated with biotinylated anti–guinea pig IgG for 1 hour, then rinsed with PBS 3 times. For immunofluorescence detection, the antibody-incubated slides were treated with PBS containing avidine–fluorescein isothiocyanate for 30 minutes and mounted using an anti-fade solution to retard fluorescence photobleaching. HE-stained or immunostained sections were photographed by Fuji super HG-100 film (Fuji Photo Film, Tokyo, Japan) using an Olympus light microscope (Olympus Optical, Tokyo, Japan) or by a digital image analyzing system using a CSU-10 confocal scanning microscope (Yokogawa Electric, Tokyo, Japan).

**RESULTS**

**MRL/lpr and NFS/s-TX Mice**

Histologic examination revealed destructive lymphocyte infiltration in lacrimal glands of 24-week-old MRL/lpr (Fig. 1A) and 10-week-old NFS/s-TX mice (data not shown). Secretory lobules were extensively replaced by aggregations of lymphocytes, especially around the secretory ducts. Acinar and duct cells of the gland showed destructive changes, including destruction of the cell membrane and degeneration. On the other hand, lymphocyte infiltration was not found in the lacrimal glands of 7-week-old BALB/c (Fig. 1B) and 10-week-old NFS/s-nonTX mice. In MRL/lpr and NFS/s-TX mice, Western blot analysis revealed that AQP5 protein had leaked into the tear fluid. As shown in Figure 1C, 28-kDa bands were specifically stained with affinity-purified anti-AQP5 IgG. AQP5 was not detected in the tear fluid of normal mice (Fig. 1C).

**NOD/Shi Jci Mice**

In NOD/Shi Jci mice, histologic examination showed lymphocyte infiltration with tissue destruction in lacrimal glands at 8 and 10 weeks but not 5 weeks (Fig. 2A). This observation shows that lymphocyte infiltration progressed with age. Immunohistochemical staining revealed the presence of AQP5 in the lacrimal glands of 5-, 8-, and 10-week-old NOD/Shi Jci and normal Jc1:ICR mice. Immunolocalization of AQP5 was observed in secretory duct cells of the lacrimal gland (Figs. 2A, 2B). By Western blot analysis, leakage of AQP5 into tear fluid was detected in 8- and 10-week-old NOD/Shi Jci mice (Fig. 2C). In contrast, AQP5 was not detected in the tear fluid of 5-week-old NOD/Shi Jci mice whose lacrimal glands showed any infiltration (Figs. 2A, 2C). In normal Jc1:ICR mice (5-, 8-, and 10-week-old), neither leakage of AQP5 into the tear fluid nor inflammatory changes in the lacrimal gland were observed (Figs. 2B, 2D). These results indicate that leakage of AQP5 in the tear is associated with age-dependent progression of lymphocyte infiltration in the lacrimal gland.

**LPS-Induced Dacryoadenitis Model Mice**

Figure 3A shows the development of inflammation in the lacrimal glands of LPS-induced dacryoadenitis model mice. At 3 hours after injection of LPS into the gland, inflammatory changes were not observed in lacrimal glands. At 9 hours, the lacrimal glands showed histopathologic features of acute inflammation. Inflammatory monocytes with neutrophils were found throughout the secretory lobules and ducts. At 24 hours, the inflammation of the lacrimal gland was developed with...
numerous neutrophils among the acinar and duct cells showing destruction or degeneration. By Western blot analysis, the AQP5 protein was found to have leaked into the tear fluid of mice killed at 9 and 24 hours (Fig. 3C). On the other hand, lacrimal glands were not damaged and AQP5 was not detected in the tears of either mice killed at 3 hours or saline-injected control mice (Figs. 3B, 3D). These observations indicate that the leakage of AQP5 protein in the tear is closely associated with lacrimal gland damage caused by LPS-induced acute inflammation.

**DISCUSSION**

In this study, we first demonstrated that AQP5 protein leaks into the tears of dacryoadenitis model mice. Lacrimal glands of these model mice showed lymphocyte infiltration and inflammation. In chronic dacryoadenitis, such as Sjögren’s syndrome, a characteristic finding is lymphocyte infiltration into lacrimal and salivary glands. In acute dacryoadenitis, biopsy specimens of the lacrimal gland show intralobular edema, lymphocytes, and plasma cell infiltration. Most lacrimal gland inflammations (acute, subacute, or chronic) are typified by lymphocytic and plasmacytic infiltrates. In Sjögren’s syndrome, proteases or cytokines produced from T cells are thought to have direct cytotoxic or destructive effects on the lacrimal gland. Histopathologic studies revealed that the salivary glands show intraepithelial penetration of lymphocytes around the ducts. In the lacrimal gland, vacuolized and edematous cells showing cytolyis were identified using electron microscopy. In this study, lacrimal cells of dacryoadenitis mice showed destructive changes. These results indicate that infiltrating lymphocytes destroy the cell membrane in lacrimal glands with Sjögren-like inflammation. In this study, we detected AQP5, which is normally localized in the apical membrane of acinar and duct cells, in dacryoadenitis mouse tear fluid. This evidence suggests that AQP5 protein leaks from damaged membranes of acinar and duct cells into the tear fluid of dacryoadenitis models.

For detection of the damage to the lacrimal gland, several methods have been proposed. Histologic evaluation of biopsy specimens of the lacrimal gland is often used to detect lacrimal gland damage. Evaluation of experimental results using computed tomography (CT) and magnetic resonance imaging of the lacrimal gland is another technique. In CT, the variability of tissues within the mixed tumor correlates with the radiologic features. It has been reported that evaluation of reflex tearing with nasal stimulation could identify two groups of patients with dry eye. Histologic evaluation of the lacrimal gland revealed that patients with poor reflex tearing were more likely to have lymphocyte infiltrations in the gland than patients with good reflex tearing. This finding suggests that the measurement of reflex tearing and the histologic evaluation of the gland are important for the diagnosis of Sicca syndrome. In the present study, we demonstrated that leakage of AQP5 protein into the tear fluid is closely associated with damage of the lacrimal gland. This evidence indicates that detection of AQP5 in tear fluid can be helpful in the diagnosis of lacrimal gland damage, thereby avoiding lacrimal biopsy. In the case of severe dry eye (e.g., Sjögren’s syndrome), it might be difficult to collect a sufficiently large volume of tear fluid for AQP5 detection, by reason of inadequate tear secretion. Having a highly sensitive method that can detect AQP5 in tear fluid will resolve this problem. In addition, we have established an enzyme-linked immunosorbent assay system for AQP5 detection in tear fluid. According to our method, the amount of AQP5 in tear fluid of dacryoadenitis mice was large enough to evaluate...
In ocular tissues other than the lacrimal gland, AQP5 is expressed in cell membranes of the corneal epithelium but not in the bulbar conjunctival epithelium or stroma. In the corneal epithelium, immunoreactivity was localized in the cell membrane of the superficial, wing, and columnar basal cells by immunohistochemistry and immunoelectron microscopy. It is possible that AQP5 leaked from injured corneal epithelium into tear fluid. However, in the present study, AQP5 was not detected in the tear fluid of control mice whose lacrimal gland showed no inflammation. Moreover, a histologic analysis of the corneal epithelium of dacryoadenitis model mice did not show any inflammation or tissue destruction (data not shown). These results suggest that AQP5 in the tear fluid is specifically leaked from the lacrimal gland and is associated with the damage of the gland. In addition, standard procedures (e.g., rose bengal or fluorescein staining of the ocular surface) may detect the corneal epithelium damage, enabling specific evaluation of the lacrimal gland damage. On the other hand, the presence of membrane proteins, such as sodium, chloride, and potassium channels or sodium/potassium adenosinetriphosphatase, has been reported not only in the lacrimal gland but also in the conjunctiva and the corneal epithelium. Moreover, to the best of our knowledge, proteins that show specific localization in the lacrimal gland have not been reported. We therefore think that proteins other than AQP5 are not useful as specific markers of lacrimal gland damage.

It has been reported that AQP2 protein excreted into urine is associated with certain disorders of the kidney. In central diabetes insipidus, urinary excretion of AQP2 occurred at a low concentration compared with healthy controls as
determined by radioimmunoassay using an anti-AQP2 antibody. In patients with impaired water excretion and hyponatremia, the urinary AQP2 concentration was higher than that in control patients under ad libitum water drinking. These results indicate that urinary AQP2 is a potent marker for diagnosing the pathologic state of central diabetes insipidus, impaired water excretion, and hyponatremia. The present study demonstrates that AQP5 leaks into the tear when lacrimal glands are damaged. In addition, immunohistochemical studies have shown that AQP5 is localized in the apical membrane of acinar and duct cells of the lacrimal gland in humans (Tsubota K, Hirai S, et al., unpublished data, 2000). Thus, we believe that detection of AQP5 in tear fluid is useful for simply and safely diagnosing lacrimal gland damage. And this method of AQP5 detection has good specificity and potential for reexamination. Moreover, AQP5 detection in the tear fluid could be used as an evaluation method for the therapeutic effects of drugs for lacrimal gland disorders with tissue destruction. However, further studies are necessary to determine whether the quantity of AQP5 protein in the tear fluid is correlated with the progression or healing of lacrimal gland damage.

In conclusion, leakage of AQP5 in the tear fluid was found to be related to lacrimal gland damage. This finding suggests that detection of AQP5 in tear fluid is useful for the specific diagnosis of lacrimal gland disorders with tissue destruction.

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References


