Abnormal Protein Profiles in Tears With Dry Eye Syndrome

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• PURPOSE: **To verify the hypothesis that protein concentrations, such as lactoferrin, epidermal growth factor (EGF), and aquaporin 5 (AQP5), in tears are abnormal in patients with dry eye.**

• DESIGN: **Prospective case-control study.**

• METHODS: **One hundred three dry eye patients were divided into three groups: dry eye not associated with the Sjögren syndrome (non-SS; n = 71), Sjögren syndrome (SS; n = 23), and Stevens-Johnson syndrome (SJS; n = 9). Sixteen normal control subjects were also checked. The concentrations of lactoerrin, EGF, and AQP5 were measured by enzyme-linked immunosorbent assay.**

• RESULTS: **The concentration of lactoferrin was significantly decreased in tears of non-SS (P = .0001), SS (P = .00005), and SJS (P = .0006) patients compared with control subjects. The concentration of EGF was significantly decreased in non-SS (P = .0005), SS (P = .00002), and SJS (P = .0001) patients compared with control subjects. The concentration of AQP5 was significantly increased in tears of only SS patients (P = .01) compared with control subjects and increased in tears of only SS patients compared with non-SS patients (P = .007).**

• CONCLUSIONS: **The decrease in both lactoferrin and EGF was found not only in SS patients but also in non-SS patients, indicating that tear components in dry eyes differ in their quantity and quality. Quantification of AQP5 increased only in SS patients, suggesting that AQP5 protein leaks into the tears when acinar cells of the lacrimal gland are damaged by lymphocytic infiltration. (Am J Ophthalmol 2003;136:291–299. © 2003 by Elsevier Inc. All rights reserved.)**

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DRY EYE SYNDROME IS CAUSED BY ABNORMALITIES in the quality or quantity of the tear film and has several important subcategories, such as dry eye not associated with Sjögren syndrome, Sjögren syndrome, and Stevens-Johnson syndrome.[1,2]

Sjögren syndrome is an autoimmune disorder, predominantly affecting women, that is characterized by decreased lacrimal and salivary gland function.[3] The lymphocytes infiltrate the epithelium of the lacrimal and salivary glands and cause cytolysis of lacrimal gland cells, resulting in severe dry eye and dry mouth.[4] Non-Sjögren syndrome dry eye includes disorders due to aqueous tear deficiency, mucin deficiency, lipid abnormalities, lid surfacing abnormalities, and epitheliopathies.[5] In patients with non-Sjögren syndrome, dry eye does not involve the lacrimal gland destruction by lymphocytic infiltration, and the gland can produce tears in response to strong stimuli.[6] The periodic reflex tearing in non-Sjögren syndrome dry eye may provide tear proteins to the ocular surface, enabling normal epithelial proliferation and differentiation. It is unclear why tear production decreases in non-Sjögren syndrome patients without apparent destruction of the lacrimal gland.[7,8] Also, the quality of tears in non-Sjögren syndrome is unknown. In patients with Stevens-Johnson syndrome, erosive involvement of the mucous membrane develops as a course of the disease, resulting in heavy dryness of the eyes and deformities of the lid margins.[2,9]

The human lacrimal gland produces tear proteins, such as lactoferrin and epidermal growth factor (EGF), which have presumed biologic activity on the ocular surface. Lactoferrin is mainly secreted from the acini of the lacrimal gland[10] and possesses antibacterial[11] and scavenging free ions.[12] Epidermal growth factor (EGF) has a

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TABLE 1. Characteristics of Study Patients

Diagnosis	Gender	n	Age	Mean Age	Age Range	Schirmer test (mm)
None	Male	4	30 ± 3	30 ± 4	28–34	27.6 ± 10.4
	Female	12	31 ± 5		20–37	
Non-SS	Male	16	39 ± 15	48 ± 16	20–68	14.1 ± 9.5
	Female	55	52 ± 15		18–82	
SS	Male	1	62	58 ± 10	62	4.8 ± 2.9
	Female	22	58 ± 10		40–75	
SJS	Male	4	42 ± 12	52 ± 14	31–58	12.4 ± 9.8
	Female	5	59 ± 11		45–75	

Age and Schirmer test are expressed as mean ± SD.
None = normal control subjects; Non-SS = dry eye not associated with Sjögren syndrome; SS = Sjögren syndrome; SJS = Stevens-Johnson syndrome.

potential regulatory role for the gland in maintaining the ocular surface, control of corneal wound healing, and diseases of the ocular surface.[13,14] Decreased EGF was also noted in Sjögren syndrome patients compared with normal control subjects.[15]

Aquaporin 5 (AQP5), which is a selective water channel protein, is highly expressed in lacrimal and salivary glands. AQP5 is normally expressed in the apical membrane of acinar and ductal cells in mice[16] and humans.[17] Distribution of AQP5 is altered in the lacrimal glands of patients with Sjögren syndrome.[17,18] Aquaporin 5 is retained in the cytoplasm of the lacrimal glands with Sjögren syndrome, which suggests a selective defect in AQP5 trafficking from cytoplasm to membrane. It is thought that rapid water transport is mediated by AQP5 in the apical membrane of the lacrimal gland.

In this prospective case-control study, we evaluated qualitatively these tear components (lactoferrin, EGF, and AQP5) in normal control subjects and in dry eye patients with non-Sjögren syndrome, Sjögren syndrome, or Stevens-Johnson syndrome. We then analyzed the correlation between the concentration of each protein and clinical indices for dry eye, tear function index (TFI),[19] rose bengal scores,[20,21] or Schirmer test. We also hypothesized that AQP5 leaked into the tears, as evidenced by destruction of infiltrated lymphocytes, resulting in poor reflex tearing. We evaluated whether the measurement of AQP5 was the optical parameter for Sjögren syndrome.

METHODS

ALL SUBJECTS PARTICIPATED IN THE SUBSPECIALTY CLINIC of the Department of Ophthalmology at Tokyo Dental College. Informed consent was obtained from all patients, and institutional review board or ethics committee approval was not required for this study.

TABLE 2. Summary of Quantification of Lactoferrin, Epidermal Growth Factor (EGF), and Aquaporin 5 (AQP5)

	Control	Non-SS	SS	SJS
Lactoferrin (mg/ml)	2.05	0.69	0.13	0.26
SD	1.12	0.55	0.22	0.33
n	15	31	15	7
EGF (ng/ml)	5.09	2.30	0.58	0.32
SD	3.74	3.04	0.60	0.16
n	17	22	12	8
Aquaporin 5 (pg/μl)	31.1	60.5	124.1	27.7
SD	23.9	105.5	137.9	25.7
n	16	65	22	7

Non-SS = dry eye not associated with Sjögren syndrome; SS = Sjögren syndrome; SJS = Stevens-Johnson syndrome.

All the experiments in this study were conducted according to the protocol approved by the tenets of the Declaration of Helsinki. The characteristics of study patients are presented in Table 1. One hundred three dry eye patients were divided into three groups: 71 non-Sjögren syndrome, 23 with Sjögren syndrome, and 9 Stevens-Johnson syndrome patients. Sixteen healthy volunteers also participated as control subjects. (The actual number of each analysis was different; see Table 2) The diagnosis of dry eye was made using the following criteria as previously reported:[22] (1) symptoms of dry eye, (2) abnormalities of test dynamics determined by the Schirmer (<5 mm after 5 minutes) and clearance tests (<8 ×), and (3) abnormalities of the ocular surface determined by rose bengal or fluorescein vital staining (>3+). The diagnosis of Sjögren syndrome was based on the criteria proposed by Fox and associates,[23] and the remaining dry eye patients with symptoms and positive findings in tear function tests or staining scores were diagnosed as non-Sjögren syndrome.

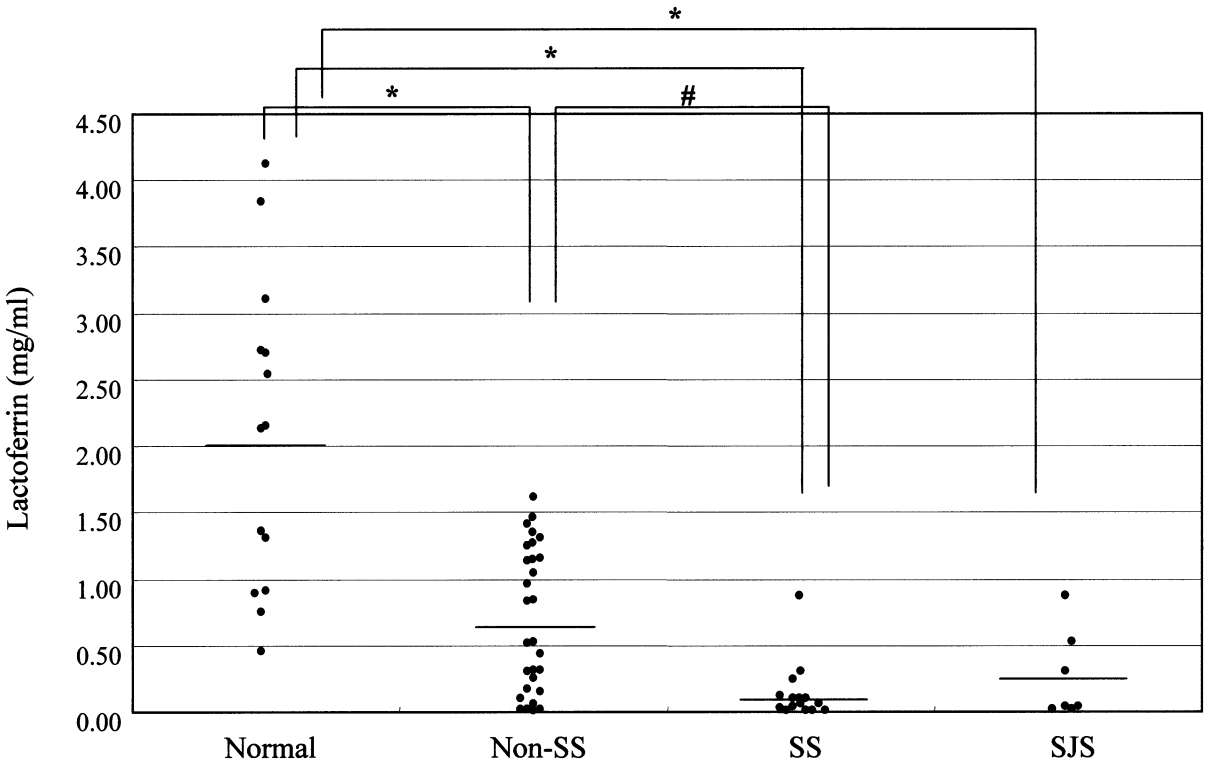

FIGURE 1. Lactoferrin concentration of tears from normal control subjects (Normal) and patients with non-Sjögren syndrome (Non-SS), Sjögren syndrome (SS), and Stevens-Johnson syndrome (SJS). Each bar in the graph shows mean values. *$P < .05$ compared with control subjects (Mann-Whitney test). #$P < .05$ compared with patients with non-Sjögren syndrome (Mann-Whitney test).

Basal tears were collected from the inferior marginal tear strip of patients with a calibrated capillary tube. Tear samples were diluted into phosphate-buffered saline (PBS) containing 0.005% t-Octylphenoxypolyethoxyethanol and stored at −80 degrees until further examination. The tear volume of 100, 500, and 400 nl was used to measure lactoferrin, EGF, and AQP5, respectively.

The concentrations of lactoferrin and EGF were measured by commercial enzyme-linked immunosorbent assay (ELISA). Assays were performed as recommended by the manufacturers. The sensitivity of the lactoferrin and EGF concentrations was above 1.6 ng/ml and 3.9 pg/ml, respectively.

All studies were undertaken with affinity-purified AQP5 antibodies. Polyclonal antipeptide antibodies specific for human AQP5 were prepared as described.[24] A synthetic peptide corresponding to the carboxy-terminus of human AQP5 (NH2-CEPDEDWEEQREERKKTMELTTR-COOH) was coupled to keyhole limpet hemocyanin (KLH) and used to immunize New Zealand white rabbits. Antihuman AQP5 immunoglobulin (Ig)G was affinity-purified from serum using a coupling gel conjugated with the synthetic peptide.

The concentration of AQP5 protein was measured by an original human AQP5 ELISA procedure, performed as follows. Human AQP5 was detected by using a competitive inhibition method. First, a 96-well ELISA Type-A plate was coated with AQP5 C-terminal peptide and incubated with a blocking reagent. Next antihuman AQP5 antibodies were reacted with a tear sample, and the combination was added to the plate coated with AQP5 C-terminal peptide. After washing with PBST (0.05% Tween 20 in PBS), the plate was incubated with antihuman AQP5 antibodies and washed again. The plate was incubated with biotinylated antirabbit Ig antibodies, washed again, and then incubated with streptavidin-biotinylated horseradish peroxidase complex. Peroxidase activity was visualized by reaction with o-phenylendiamine and H_2O_2, and the absorbance at 490 nm was measured using a microplate reader. The expression level of AQP5 in the sample was calculated from the standard curve generated with AQP5 C-terminal peptide. The sensitivity of AQP5 concentration was above 0.078 ng/ml.

• **STATISTICAL ANALYSIS:** Quantitative data are presented as mean ± standard deviation (SD). The Mann-Whitney test was used to compare the ELISA results between groups. Spearman correlation coefficients were calculated for the correlations between quantification of

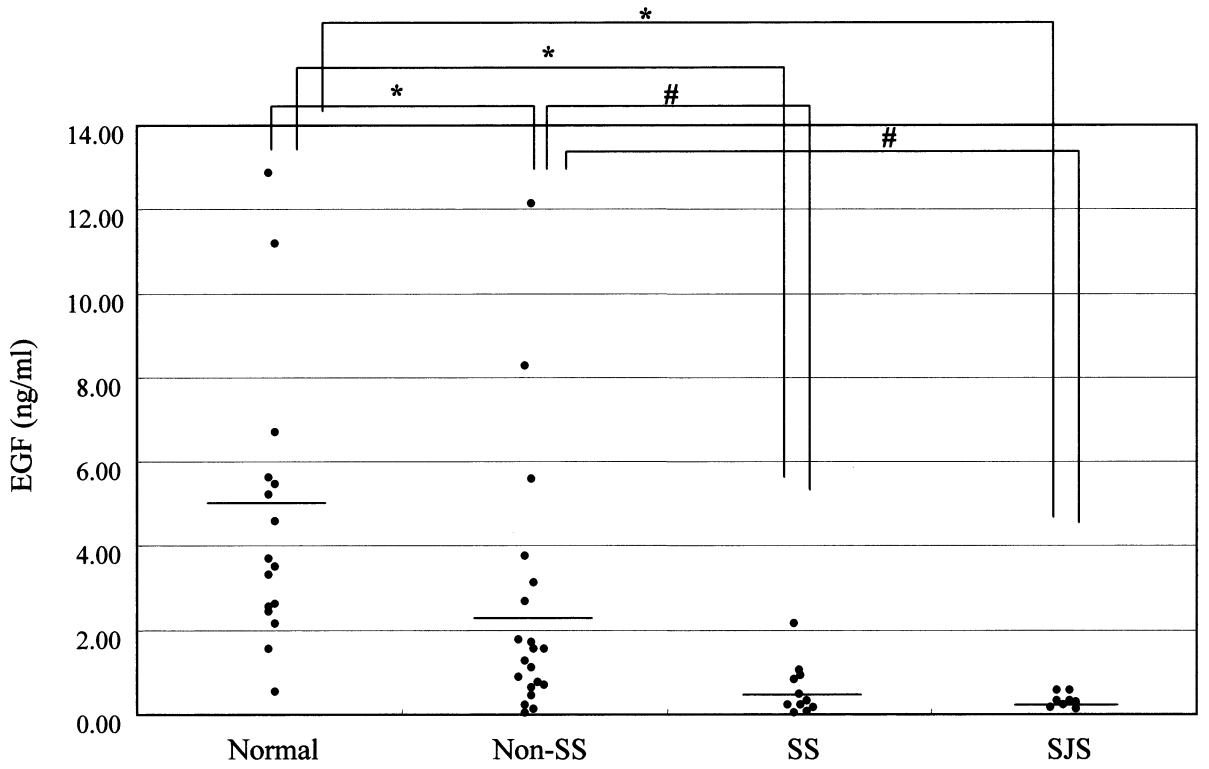

FIGURE 2. Epidermal growth factor (EGF) concentration of tears from normal control subjects (Normal) and patients with non-Sjögren syndrome (Non-SS), Sjögren syndrome (SS), and Stevens-Johnson syndrome (SJS). Each bar in the graph shows mean values. *P < .05 compared with control subjects (Mann-Whitney test). #P < .05 compared with patients with non-Sjögren syndrome (Mann-Whitney test).

proteins and clinical indices for dry eye. *P* values less than .05 were considered statistically significant.

RESULTS

LACTOFERRIN CONCENTRATION WAS MEASURED BY ELISA. Mean concentration of lactoferrin was 2.05 ± 1.12 mg/ml (n = 15) in control subjects, 0.69 ± 0.55 mg/ml (n = 31) in non-Sjögren syndrome patients, 0.13 ± 0.22 mg/ml (n = 15) in Sjögren syndrome patients, and 0.26 ± 0.33 mg/ml (n = 7) in Stevens-Johnson syndrome patients. The concentration of lactoferrin was significantly decreased in tears of patients with non-Sjögren syndrome (*P* = .0001), Sjögren syndrome (*P* = .00005), and Stevens-Johnson syndrome (*P* = .0006) compared with control subjects and decreased in tears of patients with Sjögren syndrome (*P* = .0005) compared with non-Sjögren syndrome patients (Figure 1, Table 2).

Epidermal growth factor concentration of tears was measured by ELISA. Mean concentration of EGF was 5.09 ± 3.74 ng/ml (n = 17) in control subjects, 2.30 ± 3.04 ng/ml (n = 22) in non-Sjögren syndrome patients, 0.58 ± 0.60 ng/ml (n = 12) in Sjögren syndrome patients, and 0.32 ± 0.16 ng/ml (n = 8) in Stevens-Johnson syndrome patients. The concentration of EGF was significantly decreased in tears of patients with non-Sjögren syndrome (*P* = .0005), Sjögren syndrome (*P* = .00002) and Stevens-Johnson syndrome (*P* = .0001) compared with control subjects and decreased in tears of Sjögren syndrome patients (*P* = .02) and Stevens-Johnson syndrome (*P* = .01) compared with non-Sjögren syndrome patients (Figure 2, Table 2).

Aquaporin 5 concentration of tears was measured by an indirect ELISA. Mean concentration of AQP5 was 31.1 ± 23.9 pg/μl (n = 16) in control subjects, 60.5 ± 105.5 pg/μl (n = 65) in non-Sjögren syndrome patients, 124.1 ± 137.9 pg/μl (n = 22) in Sjögren syndrome patients, and 27.7 ± 25.7 pg/μl (n = 7) in Stevens-Johnson syndrome patients. The concentration of AQP5 was significantly increased in tears of only Sjögren syndrome patients (*P* = .01) compared with control subjects and increased in tears of only Sjögren syndrome patients (*P* = .007) compared with non-Sjögren syndrome patients (Figure 3, Table 2).

The correlation between the concentration of each protein, lactoferrin, EGF, or AQP5 and TFI was analyzed. Tear function index is calculated by multiplying the

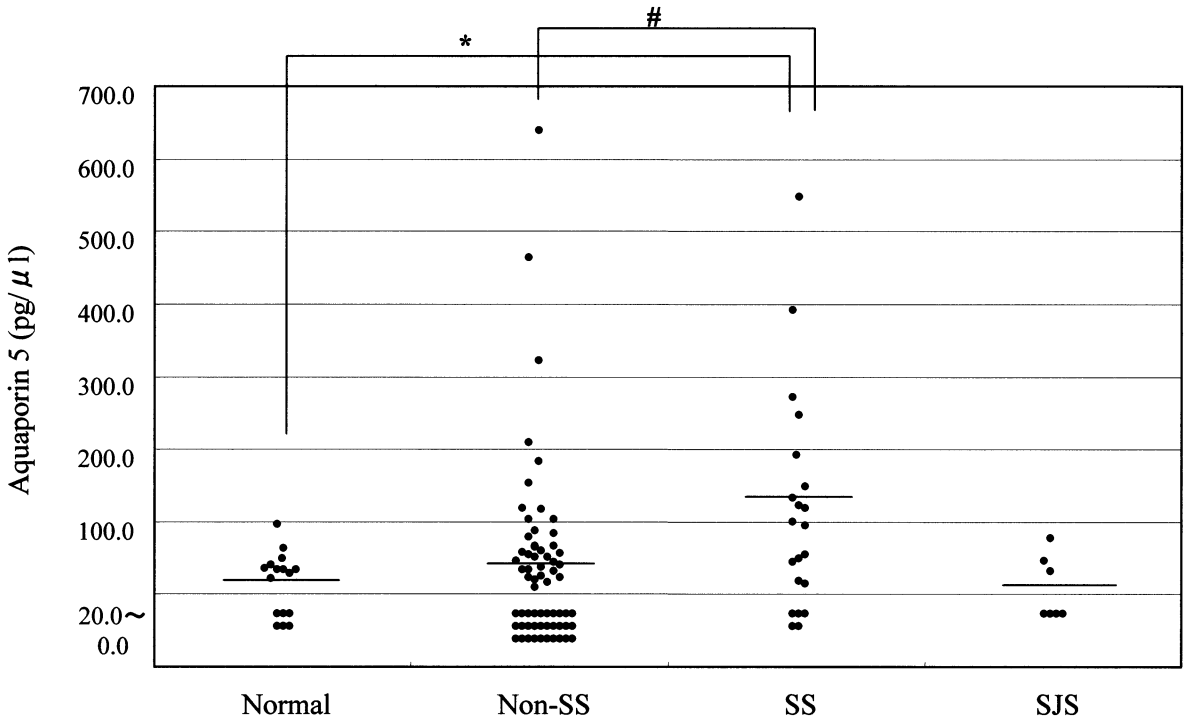

FIGURE 3. Aquaporin 5 (AQP5) concentration of tears from normal control subjects (Normal) and patients with non-Sjögren syndrome (Non-SS), Sjögren syndrome (SS), and Stevens-Johnson syndrome (SJS). Each bar in the graph shows mean values. *$P < .05$ compared with control subjects (Mann-Whitney test). #$P < .05$ compared with patients with non-Sjögren syndrome (Mann-Whitney test).

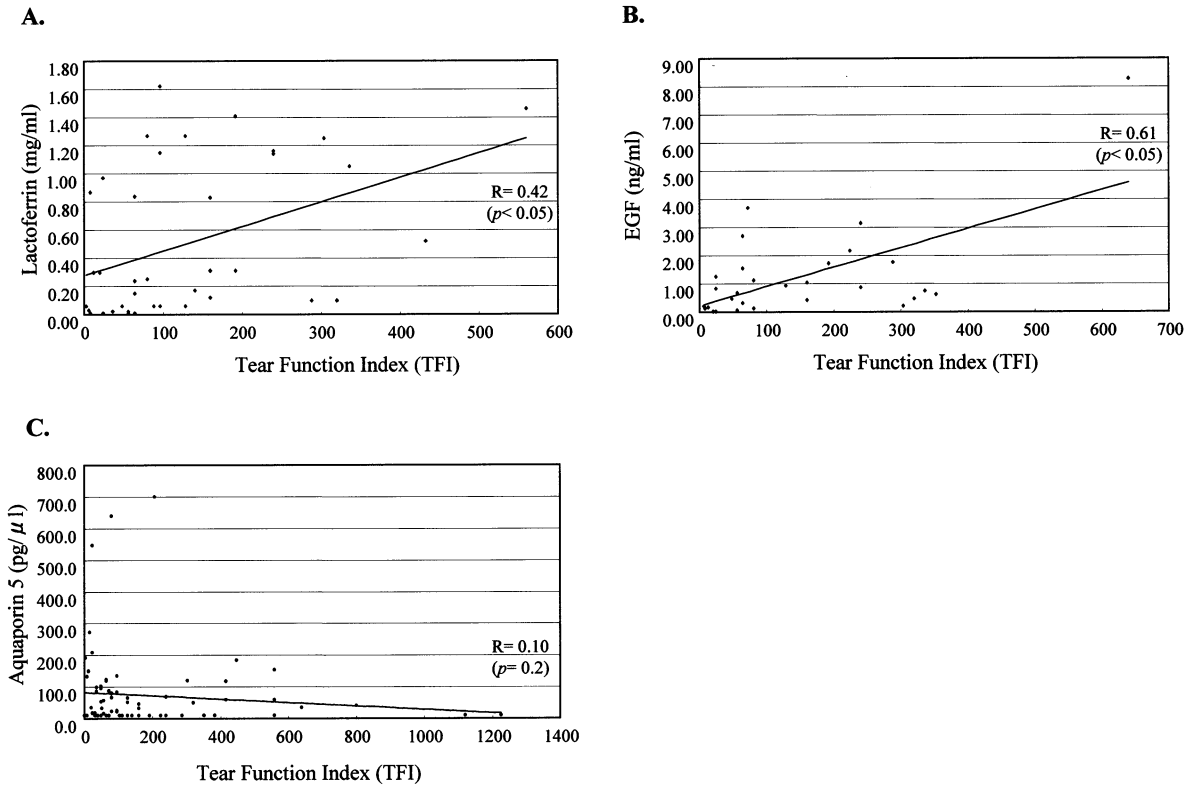

FIGURE 4. (A) Correlation between lactoferrin concentration in tears and tear function index (TFI). (B) Correlation between epidermal growth factor (EGF) concentration in tears and TFI. (C) Correlation between aquaporin 5 (AQP5) concentration in tears and TFI. Spearman correlation coefficients were calculated for the correlations between quantification of each protein and TFI.

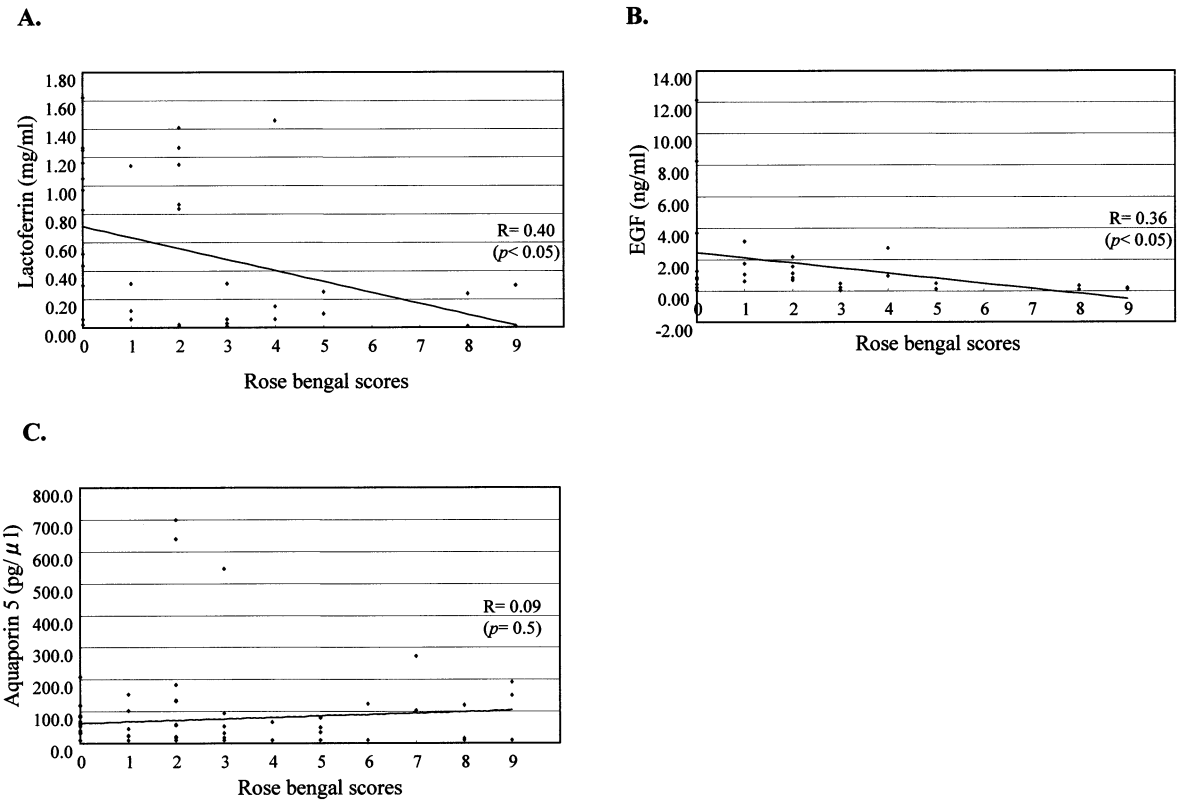

FIGURE 5. (A) Correlation between lactoferrin concentration in tears and rose bengal scores. (B) Correlation between epidermal growth factor (EGF) concentration in tears and rose bengal scores. (C) Correlation between aquaporin 5 (AQP5) concentration in tears and rose bengal scores. The Spearman correlation coefficients were calculated for the correlations between quantification of each protein and rose bengal scores.

Schirmer test value by the tear clearance rate. Correlations between both lactoferrin ($P = .0008$) or EGF ($P = .01$) and TFI were statistically significant (Figure 4, A and B), whereas correlation between AQP5 and TFI was not ($P = .2$; Figure 4C).

The correlations between the concentration of each protein, lactoferrin, EGF, or AQP5 and rose bengal score were analyzed. Correlations between both lactoferrin ($P = .006$) and EGF ($P = .01$) and rose bengal scores were statistically significant (Figure 5, A and B), whereas the correlation between AQP5 and rose bengal scores was not ($P = .5$; Figure 5C).

The correlations between the concentration of each protein, lactoferrin, EGF, or AQP5 and Schirmer test without anesthetic (Schirmer 1) were analyzed. Correlations between both lactoferrin ($P = .008$) and EGF ($P = .03$) and Schirmer test were statistically significant (Figure 6, A and B), whereas the correlation between AQP5 and Schirmer test was not ($P = .1$; Figure 6C).

The correlations between the concentration of each protein and patient's age were analyzed. Correlations between lactoferrin ($P = .5$), EGF ($P = .4$), and AQP5 ($P = .1$) and patient's age were not statistically significant (Figure 7, A through C).

DISCUSSION

WE HAVE SHOWN THAT THE CONCENTRATION OF LACTOferrin was significantly decreased in tears of patients with non-Sjögren syndrome, Sjögren syndrome, and Stevens-Johnson syndrome compared with control subjects and decreased in patients with Sjögren syndrome compared with non-Sjögren syndrome patients. The concentration of EGF was significantly decreased in patients with non-Sjögren syndrome, Sjögren syndrome, and Stevens-Johnson syndrome compared with control subjects and decreased in Sjögren syndrome patients compared with non-Sjögren syndrome patients. The concentration of AQP5 was significantly increased only in Sjögren syndrome patients compared with control subjects and increased only in Sjögren syndrome patients compared with non-Sjögren syndrome patients. Correlations between pro-

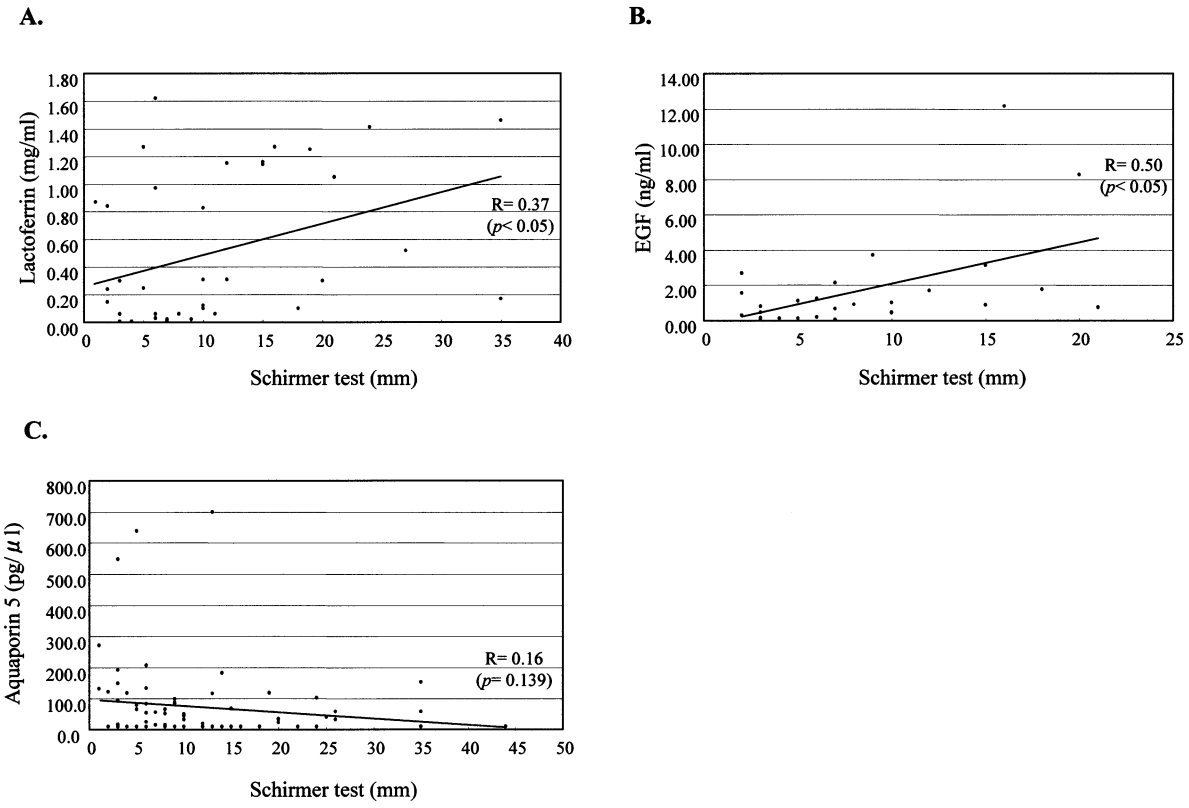

FIGURE 6. (A) Correlation between lactoferrin concentration in tears and Schirmer test. (B) Correlation between epidermal growth factor (EGF) concentration in tears and Schirmer test. (C) Correlation between aquaporin 5 (AQP5) concentration in tears and Schirmer test. The Spearman correlation coefficients were calculated for the correlations between quantification of each protein and Schirmer test.

tein concentrations and patients' ages were not statistically significant.

Tears perform a vital role in maintaining the health of the corneal epithelium. They consist of antibacterial factors, substances such as EGF, fibrinogen, basic fibroblast growth factor (bFGF), and many surface receptors.[25] We chose to study lactoferrin and EGF because they are representative of lacrimal-gland-produced proteins that can be measured by ELISA, which is a superior system in terms of sensitivity, reproducibility, and simplicity. The concentrations of lactoferrin and EGF were decreased in tears of patients with non-Sjögren syndrome, Sjögren syndrome, and Stevens-Johnson syndrome. The decrease in lactoferrin and EGF in Sjögren syndrome patients seemed to be caused by dysfunction of the lacrimal gland. Interestingly, a decrease in lactoferrin in non-Sjögren syndrome patients was also found. There are two expectations for our findings. First, the functional ability to produce tears from the lacrimal gland is abnormal under nonstimulated conditions. Also, the evaluation of reflex tearing with nasal stimulation may distinguish three types of dry eye.[26] Our evaluation demonstrates that basic tearing of patients with non-Sjögren syndrome is decreased, whereas reflex tearing remains. It has been reported that secretion of the tear proteins lactoferrin and EGF was increased by the cholinergic stimulation of the human lacrimal gland.[27] We also showed significant correlations between the concentration of lactoferrin or EGF and TFI. These results suggest that the lacrimal glands of patients with non-Sjögren syndrome have a reduced ability to produce water and tear proteins, and after stimulation with cholinergic reagents, the lacrimal gland can facilitate appropriate tear volume and proteins as it does in control subjects. Second, tear components were unstable on the ocular surface. Decreased tear volume was frequently observed in dry eye patients. Generally, in conditions of low water volume, various proteins may become proteolytic. The quantity of tears is crucial to preserving the biologic activity of the tear proteins. Stevens-Johnson syndrome patients do not automatically have dry eye, but in this study the concentrations of lactoferrin and EGF were significantly decreased in Stevens-Johnson syndrome patients. We suggest that (1) the lacrimal gland's ability to produce tears is decreased in Stevens-Johnson syndrome as well as Sjögren syndrome patients and that (2) the injured corneal epithelium of Stevens-Johnson syndrome patients

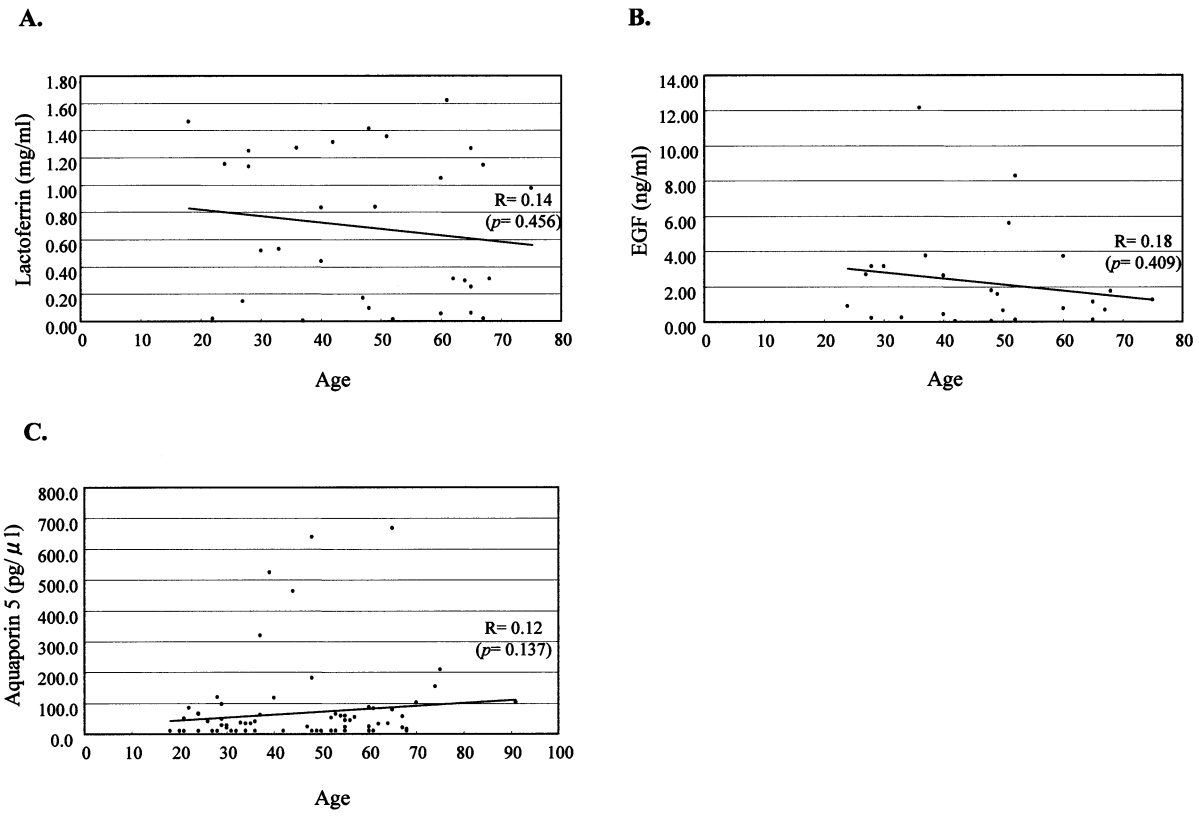

FIGURE 7. (A) Correlation between lactoferrin concentration in tears and the age. (B) Correlation between epidermal growth factor (EGF) concentration in tears and the age. (C) Correlation between aquaporin 5 (AQP5) concentration in tears and the age. Spearman correlation coefficients were calculated for the correlations between quantification of each protein and the age of the patients.

lacks the ability to preserve biologic activity of the tear proteins.

For dry eye therapy, artificial tears are required to replace a reduced or inadequate supply of natural tears. Our results suggest that tears of non-Sjögren syndrome patients are different from those of control subjects both quantitatively and qualitatively. As therapy for dry eye patients, it is insufficient to treat with artificial tears alone; the replacement of tear proteins with biologic activity is also needed. This is verified by using a rabbit short-term dry eye model in which lactoferrin reduced the loss of corneal epithelial integrity.[28] Autologous serum application is also efficient in providing essential components to the ocular surface.[29]

In Sjögren syndrome patients, the lymphocytes infiltrate into the epithelium of the lacrimal gland.[4] It has been reported that poor reflex tearing is associated with lymphocytic infiltrations in the lacrimal gland.[30] Quantification of AQP5 of the lacrimal gland showed no differences among control subjects and patients with non-Sjögren syndrome and Sjögren syndrome.[17] This suggests that patients with Sjögren syndrome continue to produce AQP5 despite the lymphocytic destruction of the lacrimal gland. In this study, quantification of AQP5 eluted into tears was significantly increased in Sjögren syndrome patients compared with control subjects. This result corresponds to the report that leakage of AQP5 in the tear was related to the lacrimal gland damage in experimental dacryoadenitis models.[31] It is unclear whether this is due to a different pathogenic mechanism in Sjögren syndrome or is merely a matter of the degree of severity of its involvement in non-Sjögren syndrome and the degree of lacrimal acinar cell dysfunction. Recent evidence suggests that the conjunctiva of patients with non-Sjögren syndrome and Sjögren syndrome exhibited infiltrating lymphocytes and evidence of markers of immune activation and inflammation, which suggests that the factors that impair lacrimal gland secretion in both non-Sjögren syndrome and Sjögren syndrome may have a common pathophysiology.[32] Aquaporin 5 is also expressed in the cell membranes of the corneal epithelium.[33] The increase of AQP5 content in some patients with non-Sjögren syndrome can be caused by an injured corneal epithelium, resulting in AQP5 elution into tears.

We have shown significant correlations between lactoferrin and EGF and clinical indices, TFI, rose bengal

scores, and Schirmer tests. Correlations between AQP5 and clinical indices were not significant, however. These differences suggest that lactoferrin and EGF produced by the lacrimal gland directly preserve ocular surface function, whereas AQP5 has not a direct but a secondary influence on ocular surface disorder, caused by a decrease in the number of lacrimal gland cells.

In this study, although the measurement of AQP5 significantly increased in Sjögren syndrome patients, independent evaluation of AQP5 may be clinically insufficient to diagnose Sjögren syndrome because deviation is considerable. The condition of the ocular surface disorder is known to measure the concentration of lactoferrin and EGF, thus combined evaluation of lactoferrin, EGF, and AQP5 may be a useful parameter for Sjögren syndrome.

In conclusion, the decrease in both lactoferrin and EGF was found in both Sjögren syndrome and non-Sjögren syndrome patients, indicating that tear components in dry eye patients with these syndromes differ in their quantity and quality. Quantification of AQP5 increased only in Sjögren syndrome patients, suggesting that AQP5 protein leaks into the tears when acinar cells of the lacrimal gland are damaged by lymphocytic infiltration.

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