Changes in Tear Protein Profile in Patients With Conjunctivochalasis

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Purpose: To compare the protein profiles of tears from normal volunteers and patients with conjunctivochalasis (CCH), with a view to identifying proteins whose expression is altered in this pathology.

Methods: Tears from 8 normal subjects and 6 patients with CCH were analyzed by 2-dimensional electrophoresis. Total protein from tears was separated in the first dimension by isoelectric focusing, and the second dimension was carried out using 8%–16% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gel images were analyzed using Progenesis SameSpot software. Those spots of interest were manually cut out from the gels, and the corresponding proteins were identified by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF). Expression levels of proteins that had been found to be significantly altered were further verified by Western blot.

Results: Approximately 250 spot proteins were detected in the whole tear proteome. Twenty-four spots were significantly upregulated in CCH compared with that in controls. Eleven protein spots were identified, which included proteins belonging to the S100 family (A8, A9, A4; 2.44, 1.71, and 2.82 fold upregulation, respectively), guanosine triphosphate–binding protein 2 (1.95 fold), L-lactate dehydrogenase A–like 6B (2.32 fold), fatty acid–binding protein (2.01 fold), keratin type I cytoskeletal 10 (1.81 fold), glutathione S-transferase P (2.27 fold), peroxiredoxin-1, peroxiredoxin-5 (1.79- and 1.92 fold, respectively), and cullin-4B+ glyceraldehyde 3-phosphate dehydrogenase (1.96 fold).

Conclusions: We have identified a group of proteins, which is upregulated in CCH tears. Although some of them, such as S100A4, S100A8, and peroxiredoxin-5, are markers of inflammation and oxidative processes, monitoring their levels in CCH might be useful for assessing the severity and progression of the disease.

Key Words: conjunctivochalasis, tears, inflammation, proteomics

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Conjunctivochalasis (CCH), defined as a redundant, loose, nonedematous inferior bulbar conjunctiva interposed between the globe and the lower eyelid, has been shown to cause different types of ocular surface irritation.[1] This disorder is a frequent cause of chronic ocular discomfort, such as irritation, epiphora, dryness, and blurred vision, and generally affects elderly people. Information regarding the pathogenesis of CCH is scanty and conflicting. Although several reports regarding the etiology of CCH have been published, there continues to be a discrepancy between the theory that emphasizes aging and ocular movement[2] and another theory that emphasizes ocular surface inflammation and delayed tear clearance as etiological factors.[3] The "aging" hypothesis presented by Hughes[4] reports the loss of subconjunctival connective tissue that provokes a loss of adherence of the bulbar conjunctiva to the sclerotic and a more superficial inferior fornix. These authors believe that there are 3 reasons that explain why the conjunctival folds are more frequent in the lower eyelid than in the upper one: gravity, the localization of the upper palpebral margin between the limbus and the center of the cornea, and finally, the anatomical difference giving rise to a larger laxity in the inferior conjunctiva in comparison to the superior one.

In contrast, Meller and Tseng believe that in addition to elastotic degeneration, a collagenolytic activity is also present that contributes to the process of CCH formation. This in turn would be exacerbated by the presence of inflammation of the ocular surface.[1] Such etiopathogenic association has also been reported in pterygium and pinguecula. Thus, CCH could itself generate a delay in tear clearance, which would lead to ocular irritation because of the accumulation of specific proteins that increase collagenolytic activity and lead to a greater relaxation of the conjunctiva.

Some authors have previously reported that proteins such as interleukin (IL)-1β and tumor necrosis factor-α regulate increased matrix metalloproteinase (MMP) expression in human cultured CCH fibroblasts.[5,6] However, to our knowledge, in vivo studies have directly explored protein expression changes in human samples, such as tears. In an earlier study performed by our group, we found that some proteins were overexpressed in CCH compared with controls; these included MMP-9, IL-1β, and IL-6, which are all involved in inflammation, degradation, and remodeling processes.[7]

In this regard, Wang et al[8] found inflammatory cytokines in tears of patients with CCH, and they concluded that inflammation may play an important role in the pathogenesis of this disease and the development of delayed tear clearance.

Clinical recognition of the pathogenic role of inflammation helps distinguish CCH from other diseases that may generate similar symptoms. However, an adequate specific therapy is yet to be determined. For symptomatic patients, topical lubricants can be tried, but they are frequently unsuccessful, and surgical excision may be required. The purpose of this study was thus to characterize the distinguishing features of the pattern of protein expression in tears from patients with CCH and determine whether tears contained specific proteins associated with the disease.

MATERIALS AND METHODS

Patients

This research was conducted by medically qualified personnel after approval by the Instituto Clínico Quirurgico de Oftalmologia Ethical Committee and in strict accordance with the tenets of the Declaration of Helsinki. Tear samples were obtained from 1 eye (always the most affected) from 23 patients (16 women and 7 men with a mean age of 65.91 ± 3.92 years) with symptomatic CCH disease manifesting itself at the lower tear meniscus. Diagnosis was based on fluorescein-assisted and lissamine green–assisted slit-lamp examination. The control tear samples were collected from one eye of 18 healthy individuals (controls) (12 women and 6 men with a mean age of 47.22 ± 9.97 years), who were not suffering from any ocular disease. Patients and normal subjects were recruited from the Cornea and Ocular Surface Unit, Instituto Clínico Quirurgico de Oftalmologia, Bilbao, Vizcaya, Spain. Informed consent was obtained from all patients after the nature and possible consequences of the study were explained.

The exclusion criteria included the presence or history of any systemic or ocular disorder or condition (including ocular surgery, trauma, and disease) that could possibly interfere with the interpretation of the results. Current or recent use of topical ophthalmic or systemic medications that could affect the pathological condition was also considered as an exclusion criterion, as well was the wearing of contact lenses and patients diagnosed with hyposecretive dry eye.

Tear Samples

For all experiments, tears were collected from the inferior lateral tear meniscus, minimizing irritation of the ocular surface or lid margin. Anesthetic drops were not instilled. We obtained tear samples using a Merocel sponge (PVA 0525; Oasis, Glendora, CA). After collection, the sponge was introduced into a 0.5-mL tube (Eppendorf, Fremont, CA) and the tear fluid was subsequently recovered by centrifugation at 13,000 rpm for 15 minutes. To perform the proteomic studies, all tear samples were cleaned with an anti-HSA/IgG Affinity Resin kit (Sartorius, Palaiseau, France) to remove some of the major proteins present in tears and increase the possibility of detecting low abundance proteins related with the disease.

Subsequently, all the cleaned samples were concentrated by using a Vivaspin 500 3000 evaporator (Sartorius) to a final volume of 50 μL approximately. Total protein concentration in each tear sample was estimated by means of the EZQ Protein Quantitation Kit (Molecular Probes; Carlsbad, CA) using bovine serum albumin (BSA) as the standard. Briefly, 1 μL of diluted tear samples in USD buffer (6-M urea, 10% sodium dodecyl sulfate [SDS], 1-mM dithiothreitol [DTT]) was applied to the assay paper. Signal intensity was recorded at 618 nm.

Isoelectric Focusing First Dimension Separation

Forty microgram of total protein from each sample was separated in immobilized pH gradient strips (11-cm format; 3–10 pH range). First dimension separation of proteins was carried out as follows: 250 V for 20 minutes followed by 8000 V for 2 hours, and finally 20,000 V/h. Once the strips were focused, an equilibration step was carried out using equilibration buffers I and II (Bio-Rad Laboratories, Hercules, CA).

Two-Dimensional Polyacrylamide Gel Electrophoresis

The second dimension SDS–polyacrylamide gel electrophoresis separation was performed using a precast Criterion 8%–16% gradient gel in a Dodeca Criterion Cell apparatus (Bio-Rad Laboratories). The gels were run at 200 V for 90 minutes. SDS and polyacrylamide gels were then stained with SYPRO Ruby (Molecular Probes), and the gel images were captured on a VersaDoc Model 4000 Imaging System (Bio-Rad Laboratories), coupled with PDQuest 2D gel image analysis software (Bio-Rad Laboratories). The images were analyzed using Progenesis software (Nonlinear Dynamics, Newcastle, United Kingdom).

Images of the same dimensions were cut and spots were detected from all gel images under the same conditions. Gel images were exported to a tiff file format for subsequent analysis using Progenesis Work Station Software (Nonlinear Dynamics). First, the images were aligned and automatically analyzed by comparing control group (healthy) versus disease group (CCH) gels using Progenesis SameSpots software. Next, the spots detected were statistically listed based on the P value for the Student t value ($P < 0.05$). Normalized volume was calculated as the estimated volume of each spot. Spot volume was defined as the optical density of a spot × the area occupied by a spot. In the analyses, percentage volume means was the volume of a spot divided by the total volume on the entire image. Differences in intensity were determined to be significant based on the normalized value. Subsequently, all protein spots showing statistically significant differences in protein expression were manually excised from the gels and subjected to protein identification.

MALDI-TOF Analyses

Spots were cut from 2-D SYPRO Ruby–stained electrophoresis gels and subjected to in-gel trypsin digestion according to Shevchenko et al[9] with minor modifications. The gel pieces were swollen in a digestion buffer containing 50-mM NH_4HCO_3 and 12.5 ng/μL of trypsin (recombinant, proteomics grade trypsin; Roche Diagnostics, Penzberg, Germany) on an ice bath. After 30 minutes, the supernatant was removed and discarded. Then, 20 μL of 50-mM NH_4HCO_3 were added to the gel piece, and the digestion was allowed to proceed at 37°C overnight. The supernatant

was transferred to an empty eppendorf tube, and basic and acidic peptide extraction was performed on the gel pieces. Supernatants of each sample were pooled and dried by vacuum centrifugation. Before mass spectrometry analysis, pellets were resuspended in 10 μL of 0.1% trifluoroacetic acid (TFA).

Recovered peptides were purified before MALDI analysis by custom-made nanocolumns as described by Gobom et al[10] with some modifications including a column consisting of 100–300 nL of POROS R2 material (PerSeptive Biosystems, Framingham, MA). The column was equilibrated with 0.1% TFA, and the bound peptides subsequently eluted directly onto the MALDI target with 0.5 μL of α-cyano-4-hydroxycinnamic acid (CHCA) solution (20 μg/μL in ACN, 0.1% TFA, 70:30 vol/vol).

Peptide mass fingerprinting was performed on a Bruker Ultraflex TOF/TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany). Positively charged ions were analyzed in reflector mode, using delayed extraction. The spectra were obtained by randomly scanning the sample surface. Typically, 600–800 spectra were averaged to improve the signal to noise ratio. Spectra were externally calibrated resulting in a mass accuracy of <50 ppm. Protein identification was performed by searching in a nonredundant protein database using the Mascot search engine (http://matrixscience.com).

Antisera

Polyclonal mouse anti-human S100A4, S100A8, and peroxiredoxin-5 antisera were purchased from Abnova Corporation (Taipei, Taiwan). The goat anti-mouse IgG-HRP–conjugated secondary antiserum was also purchased from Abnova Corporation.

Western Blot

To validate altered expression of some of the proteins most highly expressed in CCH tear samples, Western blot analyses were performed. Tear proteins were separated under reducing conditions on 15% separating, 5% stacking SDS–polyacrylamide gels (Mini-ready; BioRad, Hercules, CA) at a constant voltage (200 V) for 45 minutes, in a MiniProtean electrophoresis apparatus (Bio-Rad). Twenty-five microgram of total protein from control and CCH tears were loaded into the SDS gel and run simultaneously. Resolved proteins were transferred to nitrocellulose membranes using a Mini Tank blot apparatus (Bio-Rad) at 30 mV overnight in a cool room. After blotting, the membranes were blocked in a solution containing 1× TBST [10-mM tris–HCl (pH 8.0), 150-mM NaCl, and 0.10% Tween 20] and 5% fat-free milk for 1 hour. Subsequently, the membranes were incubated with the respective primary antisera (S100A4, S100A8, and peroxiredoxin-5; 1:200 dilution) with 1% fat-free milk overnight at 4°C. After that, the membranes were washed in 1× TBST and were incubated with the peroxidase-conjugated secondary antibody (1:5000 dilution) in 1% fat-free milk. Binding was detected using a chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL), which was exposed to x-ray film (Eastman Kodak, Rochester, NY) from 30 seconds to 2 minutes.

Statistical Analysis

The significance of spot differences was calculated according to the Student *t* test with a confidence interval at 95%; a *P* value of less than 0.05 was considered significant. The mean values obtained in proteomic studies are based on 3 replicates of each gel.

RESULTS

The proteomics analyses were made on 6 patients with CCH and 8 control individuals to investigate possible changes in protein profiles to identify specific proteins involved in the pathologic process. A total of approximately 250 protein spots was detected in the whole proteome under the experimental conditions employed (Fig. 1).

The cleanup step for albumin and immunoglobulin G indicates that the column used specifically eliminates those proteins. In the course of our study, we carried out 2-dimensional (2D) gels before and after cleanup step in tear samples of control individuals to determine the specificity of cleaning and not removing of other proteins (data not shown). All along our studies, control and CCH samples were all cleaned following exactly the same procedure.

In the proteomics analysis, one of the first steps is principal component analysis, which indicates similarities between proteomic profiles into the gels (Fig. 2). Figure 2 indicates that in general, gels corresponding to the control

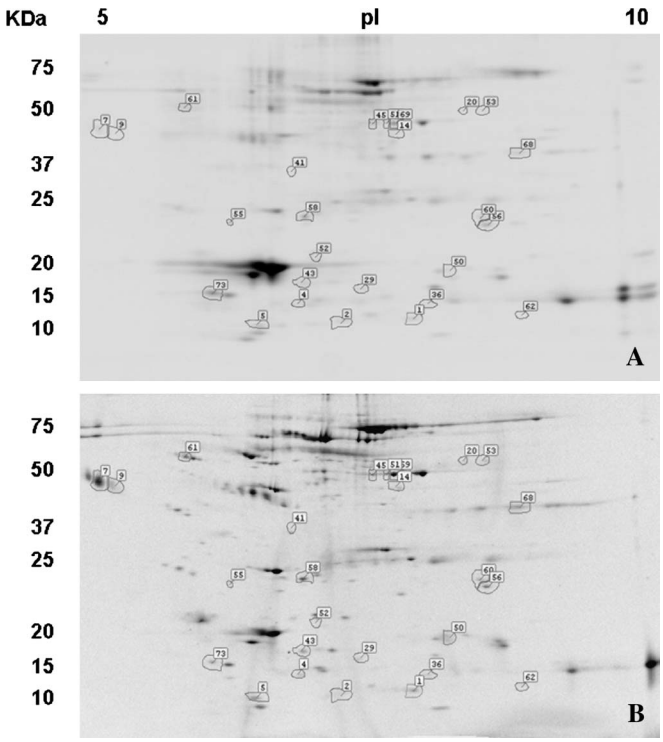

FIGURE 1. Representative SYPRO Ruby–stained 2-dimensional gels of tear proteins from a control healthy volunteer (A) and patient with CCH (B). Total protein (40 μg) from tear samples was separated on immobilized pH gradient strips (pH 3–10) in the first dimension and by 8%–16% SDS–polyacrylamide gel electrophoresis in the second dimension. All spots that were found to be altered in CCH samples with respect to control are surrounded and indicated numerically. Protein spots identified by MALDI-TOF analyses are shown in Table 1.

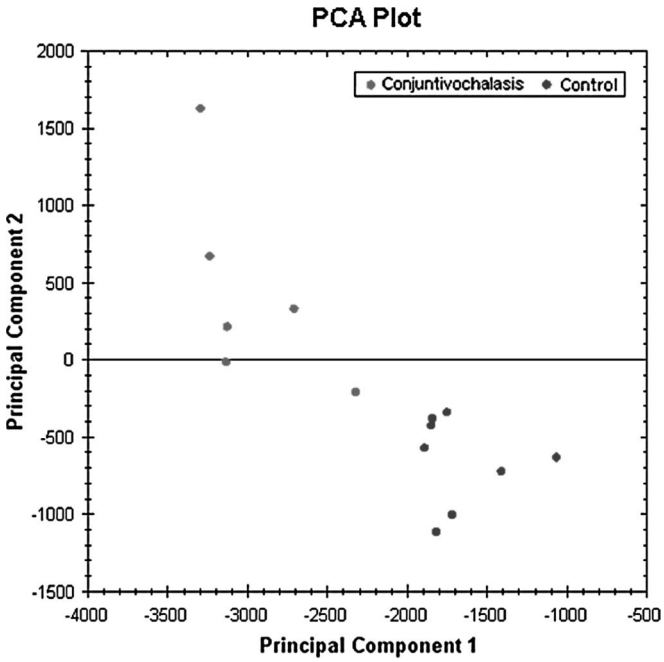

FIGURE 2. Principal component analysis of spot volume data. The figure illustrates the 2 principal components (PC1 and PC2), which explain the majority of variation in the dataset plotted against each other. Each individual gel is displayed by means of filled circles (score plot), control gels (blue), and CCH gels (red). The closer the circles, the more similar the proteomes. The plot shows how the control gels are all grouped in the lower right quadrant, whereas the CCH gels are mainly grouped in the upper left quadrant, with the exception of one gel (sample 5), which is located in the lower left quadrant. This gel was nevertheless included in the analysis given that the statistical results obtained were significant.

group (lower quadrant, blue symbols) are quite similar among themselves but different to those of patients with CCH (upper quadrant, red symbols), which in turn resemble each other.

Upon comparing the whole proteome of the CCH group with the control group, we identified a total of 26 proteins whose expression was upregulated ($P < 0.05$). However, statistical analysis indicated that only 24 of these proteins were statistically valid according to their Student t values (<0.05). These 24 proteins were subjected to MALDI-TOF identification, and we were finally able to unequivocally identify 11 of those proteins, which are listed in Table 1.

The present results (Fig. 1 and Table 1) indicate that the proteins altered in CCH with respect to control tears are all upregulated rather than downregulated. These proteins include those of the S100 family (A8, A9, and A4) (2.44 fold, 1.71 fold, and 2.82 fold, respectively), guanosine triphosphate (GTP)–binding protein 2 (1.95 fold), L-lactate dehydrogenase A–like 6B (2.32 fold), fatty acid–binding protein (2.01 fold), peroxiredoxin 1 and 5 (1.79 fold and 1.92-fold, respectively), keratin, type I cytoskeletal 10 (1.81-fold), glutathione S-transferase P (GSTP) (2.27-fold), and cullin-4B+glyceraldehyde 3-phosphate dehydrogenase (1.96-fold). Detailed analysis of 3 of the most upregulated proteins in CCH samples is presented in Figure 3. Figure 3A illustrates that in the case of S100-A8 (spot 1), increased protein expression was seen in all studied patients with CCH. A similar tendency was found for the S100-A4 (spot 5) and GSTP proteins (spot 58) (Figs. 3B, C). Statistical analyses (Student t test and fold difference index) indicate that in the case of S100-A8, S100-A4, and GSTP, there is a significant increase in protein expression in tears from patients with CCH compared with control samples (Table 1).

The histograms illustrated in Figure 4 show the ratio of protein expression levels in CCH samples in comparison to control tear samples, of all 11 identified proteins that showed statistically significant changes in protein expression as confirmed by Student t tests.

Once obtained a group of altered proteins, a confirmation step was required to validate in novel samples the protein changes observed in proteomic studies; therefore, we carried out Western blot analyses in an additional 17 patients with CCH and 10 controls. The proteins S100A4, S100A8, and peroxiredoxin 5 were chosen for these analyses because of their increased levels in the disease condition and their physiological relevance.

Results using antisera specific for each of these molecules are illustrated in Figure 5. In the case of peroxiredoxin-5, this protein band is absent in the samples from healthy control individuals, whereas a strong band is present in all 3 CCH tear samples assayed (Fig. 5A).

Similarly, changes in the expression of S100A8 and S100A4 proteins were evaluated and confirmed by Western blot under the same experimental conditions (Figs. 5B, C). The results indicate that both proteins are highly expressed in CCH tear samples in comparison to control tear samples. As shown in Figure 5B, the intensity of the S100A8 protein band (10 kDa) is significantly stronger in the CCH tear samples than in one of the control samples; in the second control sample analyzed in this assay, it is completely absent, further corroborating the results obtained in 2D proteomic studies.

A similar pattern was noted for the S100A4 protein (Fig. 5C). Thus, the level of expression of this protein is higher in the 2 CCH tear samples than in the control sample studied, in which the protein band is just perceptible. Overall, these Western blot results validate the findings obtained using the proteomics approach.

DISCUSSION

In the present study, we aimed to identify proteins whose expression is altered in the tears of patients with CCH, with respect to normal control subjects, using proteomic technology based on 2D gels. The identification of proteins specifically associated with CCH would help to advance our understanding of both the etiopathogenesis of this condition, as well as its associated symptoms, such as epiphora, pain, and in the most extreme cases, marginal corneal ulcer because of mechanical abrasion of tissue that is chronically inflamed and fragile.

The proteomic technology based on 2D gel contains a first stage of cleanup of tear samples. In the approach used in our research, we employed a cleanup step to remove major proteins and allow us to detect lower abundance proteins that normally are masked by high abundance proteins. This

TABLE 1. Expression Data and Identification of Deregulated Proteins in Tears from Patients With Conjunctivochalasis Versus Healthy Controls

Spot	Protein Name	ID	*T* test (*P*)	ANOVA (*P*)	Expression Change	Dif. Norm. Vol Fold
1	Protein S100-A8	P05109	0.000	0.000	↑	2.44
2	NI	No ID	0.000	0.000	—	—
4	Protein S100-A9	P06702	0.000	0.000	↑	1.71
5	Protein S100-A4	P26447	0.000	0.000	↑	2.82
7	NI	No ID	0.001	0.001	—	—
9	NI	No ID	0.002	0.002	—	—
14	GTP-binding protein 2	Q9BX10	0.004	0.004	↑	1.95
20	L-lactate dehydrogenase A-like 6B	Q9BYZ2	0.008	0.008	↑	2.32
29	Fatty acid–binding protein, epidermal	P05413	0.011	0.011	↑	2.01
36	NI	No ID	0.013	0.013	—	—
41	NI	No ID	0.014	0.014	—	—
43	NI	No ID	0.016	0.016	—	—
45	NI	No ID	0.016	0.016	—	—
50	Peroxiredoxin-5	P30044	0.023	0.023	↑	1.92
52	NI	No ID	0.024	0.024	—	—
53	Keratin, type I cytoskeletal 10	P13645	0.024	0.024	↑	1.81
55	NI	No ID	0.030	0.030	—	—
56	NI	No ID	0.030	0.030	—	—
58	Glutathione S-transferase P	P09211	0.030	0.030	↑	2.27
60	Peroxiredoxin-1	Q06830	0.032	0.032	↑	1.79
61	NI	No ID	0.033	0.033	—	—
62	NI	No ID	0.034	0.034	—	—
68	Cullin-4B+glyceraldehyde 3-phosphate dehydrogenase	Q13620 +P04406	0.039	0.039	↑	1.96
69	NI	No ID	0.306	0.040	—	—

ANOVA, analysis of variance; NI, not indicated; Dif. Norm. Vol., difference normalized volume.

procedure and strategy for depletion of the samples is very common in proteomics studies of other fluid proteomes such as serum/plasma.[11,12] One of the main challenges of proteomics is to find molecular signatures of biomarkers or protein disease-related. For example, the application of proteomics in the search for potential diagnostic/prognostic biomarkers in the serum of patients has been limited by the presence of highly abundant albumin and immunoglobulins that constitute approximately 60%–97% of the total serum proteins, and the remaining is assumed to include many potential biomarkers that are typically of low abundance. Therefore, removal of high-abundance proteins has become a common practice to enrich for low-abundance proteins treating the sample with affinity columns before the 2-dimensional gel electrophoresis (2-DE) analysis, allowing the visualization of low abundant proteins, and the subsequent improvement in the number of spots detected and their resolution after 2D gel.

The protein profile found in this study indicates an inflammatory event, and there is no evidence of proteins specific to CCH. Three proteins of the S100 family were overexpressed in tears from patients with CCH (S100-A8, S100-A9, and S100-A4). These proteins are known to participate in proinflammatory processes that occur at the ocular surface. S-100 A8 (calgranulin A) is a protein that is synthesized in the corneal epithelium and by stromal keratinocytes. It induces the synthesis of proinflammatory cytokines such as IL-1α. In the tears of patients with dry eye, calgranulin A has been found to be overexpressed with respect to healthy controls, and consequently, it was proposed to be a biomarker of inflammation.[13] Our results corroborate this finding by showing that a process of inflammation at the ocular surface occurs in CCH. In contrast, S100-A9 (calgranulin B) is synthesized in the interior of granulocytes and is secreted subsequently to the extracellular space. There, it exerts a proinflammatory effect and it is also involved in the migration of leukocytes. It has been found in ocular inflammatory processes in the periphery of the cornea and iris.[14] Recent studies[15,16] have validated the use of the S100A8 and S100A9 proteins as markers of inflammation. Patients with pterygium showed higher S100A8/A9 levels in tears although S100A8/A9 is a general marker for inflammation[16].

S100-A4 is a small calcium-binding protein, whose expression frequently changes during carcinogenesis. It is thought to play a role in cell proliferation.[17] It is used as a biomarker of metastasis because it is overexpressed in metastatic cells of hepatocyte carcinoma and contributes to tumor dissemination by means of inducing the synthesis of MMP-9.[18] This finding is consistent with the increased level of MMP-9 reported in tears of subjects with CCH.[7] In addition, Oslejsková et al[19] speculated that the S100A4 protein that is located at sites of inflammation, particularly at sites of joint destruction, might be implicated in the pathogenesis of chronic autoinflammatory diseases. It thus seems that the presence of conjunctival folds while occupying the lower tear meniscus leads to a delay in

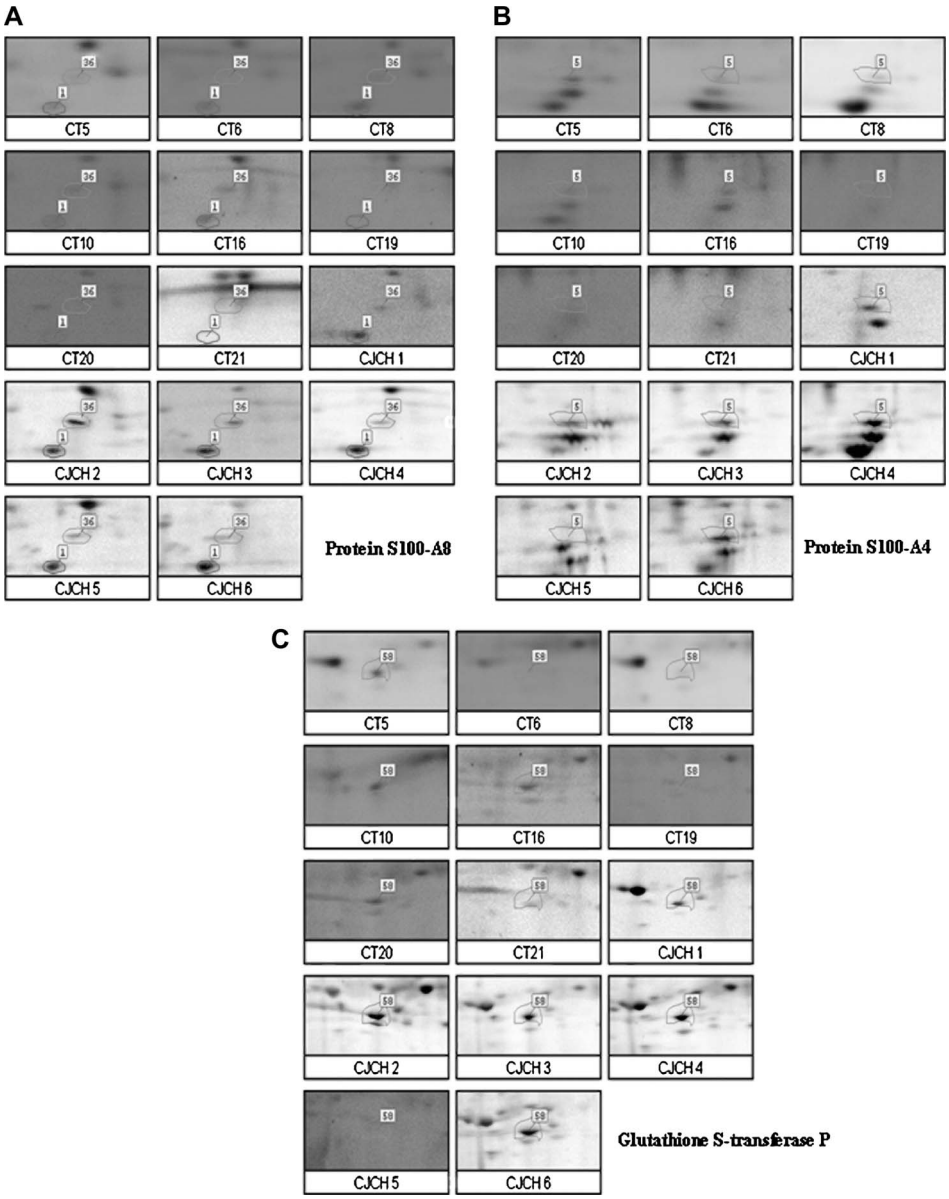

FIGURE 3. Detailed sections of 2-dimensional electrophoresis gels showing changes in protein expression in 8 control healthy volunteers (CT5, CT6, CT8, CT10, CT16, CT19, CT20, and CT21) and 6 patients with CCH (CJCH1, CJCH2, CJCH3, CJCH4, CJCH5, and CJCH6). A, Spot 1, S100A8. B, Spot 5, S100A4. C, Spot 58, GSTP.

tear clearance, which in turn leads to an alteration in tear composition because delayed tear clearance increases the residency time of the contents of the tear film and may activate or potentiate ocular surface inflammation.[20,21] In these retained tears (from our patients with CCH), we found an overexpression of proinflammatory proteins. This result suggested that in the ocular surface of patients with CCH, an inflammatory process is taking place [we have verified this affirmation using pharmacological and surgical treatments that reduced inflammation and contributes to the symptomatic relief of the patient (data not published)]. This delayed clearance could induce an overexpression of these proinflammatory proteins, thus leading to more inflammation of the ocular surface.[22]

We also identified overexpressed proteins that are implicated in the synthesis and degradation of the cytoskeleton, such as GTP-binding protein 2. Moreover, GTP-binding protein 2 is considered to be a molecular switch that regulates fundamental cell processes including growth, differentiation, and maintenance of cell structure.[23] The presence of these proteins in CCH tears may well be indicative of a role played by actin and myosin fibers, among others, in the formation of the conjunctival folds.

CCH is also characterized by the existence of severe squamous metaplasia (grade 4), in which the conjunctival epithelial cells often present a keratinized cytoplasm (depending on the degree of CCH).[24] Here, we have found overexpression of the keratin type I cytoskeletal 10 protein ($\times$1.81 fold upregulation) in samples of CCH tears. In Sjögren syndrome, this protein is related to keratinization of cells of the conjunctival epithelium.[25] For this reason, overexpression of this protein in the samples of pathological tears would indicate that in the conjunctival epithelium of these patients, a process

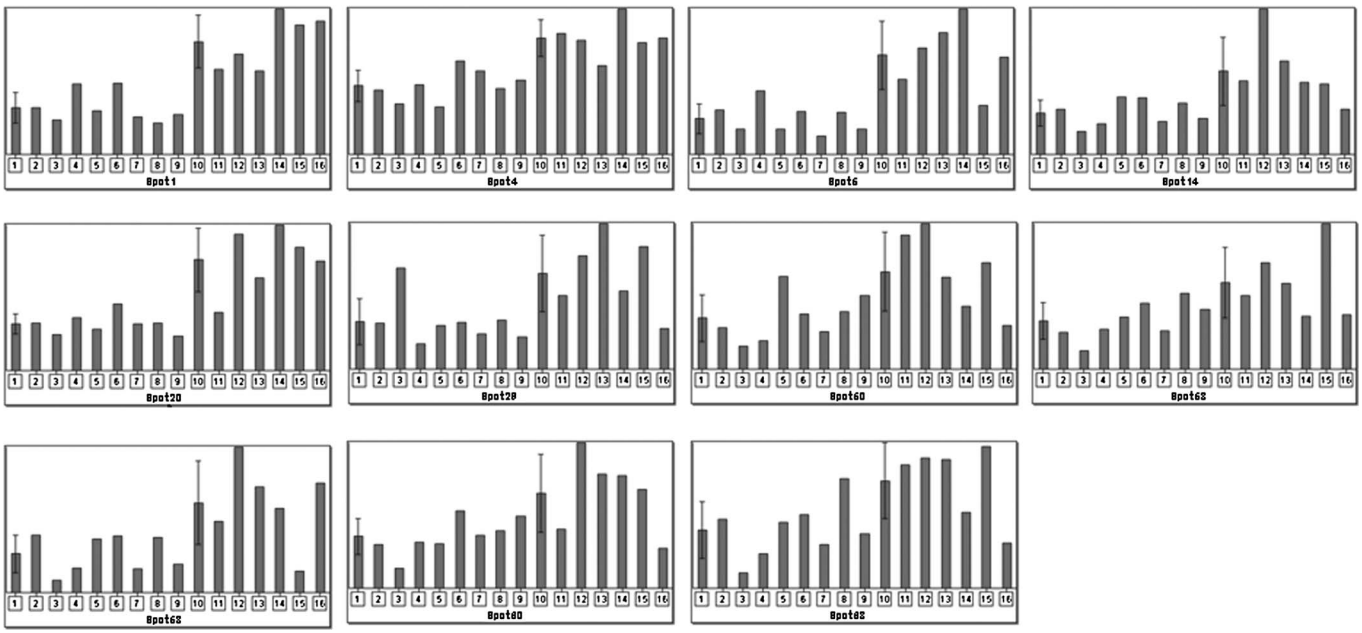

FIGURE 4. Protein expression profiles. Columns 1 and 10 indicate mean volume value for each selective spot for control and CCH samples. Columns 2–9 represent spot intensity from control samples; columns 11–16 represent spot intensity from CCH samples.

of keratinization is taking place and that the keratin type I cytoskeletal 10 protein may be implicated in this process by means of the aggregation of microfibrils. Moreover, in CCH tears, we found overexpression of proteins that are implicated in processes of hypoxia and oxidative stress, such as peroxiredoxin-1, peroxiredoxin-5, L-lactate dehydrogenase A, GSTP, and the fatty acid–binding protein. Some of these proteins play an antioxidant role and are expressed under conditions of oxidative stress as a mechanism of cell defense. An increase in L-lactate dehydrogenase A activity can be interpreted in 2 ways. First, it could indicate the existence of tissue stress because of hypoxia because L-lactate dehydrogenase A is a target of the hypoxia inducible factor-1 alpha, which induces fermentative glycolysis under these conditions.[26] Alternatively, the increased levels of this enzyme could provide a protective response to oxidative stress, by preventing intoxication and cell death because of the nitric oxide–free radical.[27]

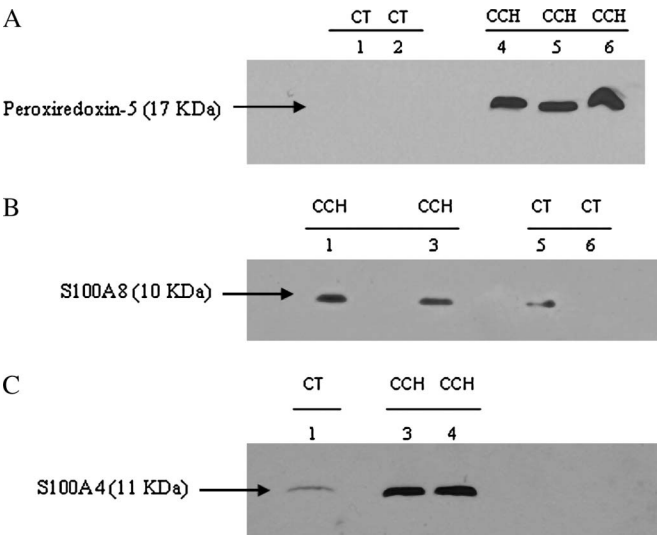

FIGURE 5. Western blot analysis of the protein expression of S100A4, S100A8, and peroxiredoxin-5 in tear samples from patients with CCH versus healthy control individuals (CT), all analyzed under the same experimental conditions. Membranes were incubated overnight at 4°C with each specific primary antiserum (1:200) and with the goat anti-mouse IgG-HRP–conjugated secondary antiserum (1:5000) for 1 hour at room temperature.

GSTP, which we found to be upregulated in CCH, belongs to a family of enzymes that are known to be expressed for the deintoxication of products synthesized after oxidative processes occurring in the conjunctival epithelium during CCH.[28] Thus, in this epithelium, the synthesis of reactive oxygen species such as H_2O_2 would lead to the upregulation of GSTP, which then acts by stimulating the expression of proteins implicated in the antioxidant response.

Peroxiredoxins are proteins that are predominantly found in the interior of the cell, although they can also be secreted. Peroxiredoxin 5 is principally known for its protective function via its antioxidant activity because it can reduce hydrogen peroxide and alkaline hydroxyperoxides. Peroxiredoxin-5 exerts its protector function against inflammatory processes in different tissues.[29] In the specific case of CCH, peroxiredoxin 5 may be catalyzing oxidative products in tears derived from the inflamed conjunctival epithelium.

Finally, a recently discovered protein, known as cullin 4B,[30] was found to be overexpressed in our samples of pathological tears. This protein belongs to the family of ubiquitinases, which acts as ligands of hormones and atmospheric toxins, by means of forming complexes that determine the protein to be degraded. At present, it is unclear what function

this protein may be playing in CCH. Additionally, it is necessary to determine the expression of this protein in further studies given that in the present work, it was resolved as a protein that comigrated with the glyceraldehyde 3-phosphate dehydrogenase protein, both being identified as a mixture in spot 68.

For validation of protein changes detected by proteomics studies, we selected the S100A4 and S100A8 proteins because they were the 2 most highly expressed proteins in CCH tears and they are known to play a role in inflammatory processes. Additionally, we also selected the peroxiredoxin-5 protein mainly because of its antioxidant activity and protective role in inflammatory process. Western blot assays corroborated the results obtained by proteomic studies.

Overall, this work has revealed the potential usefulness of analyzing the protein components of tears as a source of information about the pathogenic mechanisms implicated in ocular surface disorders. In the specific case of CCH, it seems that processes of inflammation, keratinization, and oxidative stress play an important role. Because tear clearance is altered in this disorder, we cannot discern whether our results are related to the pathogenesis or to the consequence of the tear changes, such as hyperosmolarity. It seems appropriate to consider that a vicious circle could be established between the altered tear fluid and the conjunctival disorder.

Moreover, one limitation of this study was the difference in age population between groups (65 CCH group vs. 45 control group). It is because of the difficulty to find and recruit elderly subjects devoid of any ocular surface disease to include in the control group. This fact could affect in any sense the results obtained through proteomics studies; therefore, to confirm the changes observed in tear proteome, a validation step was included in the present study. Nevertheless, further validation studies in elderly population (older than 60 years) are recommended to prove the findings presented in this work. Besides, in future investigations, it should be interesting to examine the levels of these proteins in other ocular surface disorders and analyze the degree of overexpression of these proteins in different clinical degrees of CCH, with a view to evaluating the prognostic value of identified proteins.

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