Proteomic analysis in pterygium; upregulated protein expression of ALDH3A1, PDIA3, and PRDX2

Sun Woong Kim,[1] Jonghoon Lee,[2] Boram Lee,[2] Taiyoun Rhim[2]

[1]Department of Ophthalmology, Hando Hospital, Ansan, Korea; [2]Department of Bioengineering and Institute for Bioengineering and Biopharmaceutical Research, Hanyang University, Seoul, Korea

Purpose: To identify differentially expressed proteins in the pterygium compared to healthy conjunctiva using a proteomic analysis.

Methods: Pterygial and healthy conjunctival tissues were obtained from 24 patients undergoing pterygium excision. Total proteins of the pterygia and healthy conjunctiva were analyzed with one-dimensional electrophoresis, and protein bands of interest were excised and subjected to liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-MS/MS) using Thermo's Finnigan ProteomeX workstation LTQ linear ion trap MS/MS. Using bioinformatics, differentially expressed proteins were classified, and three proteins closely involved in the response to oxidative stress were selected for further validation. Differential expression of these proteins was confirmed with western blot and immunohistochemistry.

Results: A web-based gene ontology program, DAVID, was used to classify 230 proteins that were differentially expressed in pterygial tissues. Among these genes, we chose three proteins, aldehyde dehydrogenase, dimeric NADP-preferring (ALDH3A1), protein disulfide-isomerase A3 (PDIA3), and peroxiredoxin-2 (PRDX2), that were significantly upregulated in pterygium and further increased in recurrent pterygium. Immunohistochemistry and western blot analysis confirmed that these three proteins were mainly detected in the basal epithelial layer, and their expression was significantly increased in the pterygium compared to normal conjunctiva.

Conclusions: This study reported increased expression of ALDH3A1, PDIA3, and PRDX2 in pterygia using a proteomic approach. These proteins are presumed to have a protective role against oxidative stress-induced apoptosis. This result is consistent with the hypothesis that oxidative stress is a significant factor in the pathogenesis of pterygia.

Pterygium refers to a wing-shaped fibrovascular tissue thought to be formed by the overgrowth of transformed conjunctival tissue into the cornea. The lesion is characterized by centripetal growth of a leading edge of altered limbal epithelial cells, followed by squamous cell metaplasia with goblet cell hyperplasia. Activated stromal fibroblasts, neovascularization, and extracellular matrix remodeling are also present [1-4].

Ultraviolet (UV) radiation damage, chronic irritation, and/or inflammation are hypothesized to play roles in the pathogenesis of pterygium. Patients with increased ultraviolet (UV) exposure, such as those living close to the equator or those with sunlight-related conditions, have an increased risk of pterygia formation [3-5]. Previous studies focused primarily on the clinical characteristics and surgical management of the disease, and therefore, its pathogenesis remains incompletely understood. However, considerable progress in this area has been achieved, and recent evidence implicates antiapoptotic mechanisms, oxidative stress, immunological mechanisms, various cytokines, and extracellular matrix (ECM) modulation as possible causative factors [3,6-10]. Regardless of the inciting agent, inflammatory mediators appear to be associated with pterygia. Interleukin (IL-6), IL-8, and tumor necrosis factor-α are increased in pterygia and induced after UV-B exposure in cultured pterygia epithelial cells [11]. Cyclooxygenase-2 is overexpressed in pterygia, contributing to a further increase in the inflammatory cascades [12]. Immunological mechanisms also likely contribute to the development of pterygia. An increase in mast cells, lymphocytes, plasma cells, dendritic cells, and CD4+ and CD8+ T cells in pterygia has also been documented [13]. The contribution of inflammatory mediators, immunological mechanisms, angiogenic stimulation, and ECM modulation are likely secondary in nature after actual pterygia formation [6].

In this study, we adopted a proteomics technique for identifying the proteins that were differentially expressed in pterygium compared to normal conjunctiva. Proteomics has expanded the opportunities to identify disease-specific proteins involved in many diseases, including ophthalmic diseases such as dry eye [14,15], cataract [16,17], keratoconus [18], myopia [19], and pterygia [20].

Correspondence to: Taiyoun Rhim, Department of Bioengineering and Institute for Bioengineering and Biopharmaceutical Research, Hanyang University, Seoul, Korea; Phone: +82-2-2220-2347; FAX: +82-2-2220-4347; email: rhim@hanyang.ac.kr

As in previous studies, hundreds of protein molecules were newly identified as candidate biomarkers of pterygia. To narrow our interests, we focused on proteins that show increased expression in primary pterygium and are further increased in recurrent pterygium. Among those proteins, we further validated increased expression of three proteins known to have roles related to oxidative stress, aldehyde dehydrogenase, dimeric NADP-preferring (ALDH3A1), protein disulfide-isomerase A3 (PDIA3), and peroxiredoxin-2 (PRDX2), using western blot analysis and immunohistochemistry.

METHODS

Subjects and tissue preparation: Between May 2012 and Apr 2013, 24 subjects, 10 males and 14 females, with mean age 49±5.2 (38-62) years old who underwent pterygium excision in Hando general hospital (Ansan, Korea) were included in the study. Samples of healthy conjunctiva were obtained from the same patients who underwent pterygium excision. Pterygium tissues and remnants from the autologous upper bulbar conjunctiva grafts were collected immediately at the time of surgery and frozen in liquid nitrogen. This study protocol was approved by the institutional review board of our hospital and it conformed to the guidelines provided in the ARVO statement on human subjects in ophthalmic and vision research. Written informed consent to participate in this research was obtained from each patient.

One-dimensional gel electrophoresis and LC-ESI-MS/MS analysis: Pterygia tissues were homogenized using Tissue-Ruptor (Qiagen, Valencia, CA) with PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, CA). Proteins were quantified with bicinchoninic acid (BCA) assay (Pierce, Cramlington, UK). Fifty micrograms of proteins were separated with gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 4–20% polyacrylamide gels, Novex gel, Invitrogen, Carlsbad, CA). After Coomassie staining, the protein bands of interest were excised and subjected to in-gel trypsin digestion as previously described [21]. Briefly, after destaining in 75 mM ammonium bicarbonate/40% ethanol (1:1), the gels were subjected to reducing (5 nM dithiothreitol [DTT] in 25 mM ammonium bicarbonate) and alkylating (55 mM iodoacetamide) conditions, each at room temperature for 30 min. After dehydration with acetonitrile, 20 μg/mL sequencing-grade trypsin (Roche Applied Science) containing 25 mM ammonium bicarbonate (pH 8.5) was added, and the slice was incubated at 37 °C overnight. The in-gel tryptic peptides were eluted with 0.1% formic acid. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-MS/MS) analysis was performed using Thermo's Finnigan ProteomeX workstation LTQ linear IT MS (Thermo Electron, San Jose, CA) equipped with a nanospray ionization (NSI) source (San Jose, CA).

Data-dependent acquisition mode (m/z 300–1800) was enabled, and each survey MS scan was followed by five MS/MS scans with the 30 s dynamic exclusion option on. The spray voltage was 1.9 kV and the temperature of the ion transfer tube was set at 1957C. The normalized collision energy was set at 35% [22]. MS/MS data were searched based on the IPI human protein database (version 3.29) using the SEQUEST algorithm (Thermo Electron). Scaffold ver. 01_07_00 was used to validate MS/MS-based peptides and protein identification.

Gene ontology analysis: Gene ontology analysis was performed using Integrated Discovery (DAVID) Functional Annotation Tool. Through DAVID, proteins with gene symbols were grouped according to biologic process.

Western blot analysis: Protein extracts were obtained from pterygia and normal conjunctiva tissue and were lysed by sonication in PBS containing 1 mM PMSF. Proteins were resolved with 12.5% SDS–PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Milan, Italy). The membrane was blocked for 1 h in a 5% skim milk solution and then incubated with mouse monoclonal antibodies against ALDH3A1 (1:2,000 dilution, AbFrontier, Seoul, Korea), PDIA3 (1:5,000 dilution, AbFrontier), and PRDX2 (1:2,000 dilution, AbFrontier) for 2 h at room temperature. The unbound primary antibodies were removed with one 15-min and two 5-min washes in PBS containing 0.1% (v/v) Nonidet P40. Then the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (IgG) secondary antibody (Ab Frontier) for 2 h at room temperature. The target protein was detected with enhanced chemiluminescence (ECL) solution (Amersham Biosciences, Cleveland, OH) using X-ray film.

Immunohistochemistry: The tissue was fixed with 4% (v/v) buffered paraformaldehyde for 1 day and then washed in tap water for 1 day. The tissue was dehydrated with a series of graded ethanol and embedded in paraffin. Sections were cut to a thickness of 4 μm and deparaffinized. Then, the sections were hydrated using a series of decreasing concentrations of ethanol. The sections were incubated in blocking buffer for 1 h at room temperature to block non-specific binding. The primary antibodies used were against ALDH3A1 (1:200 dilution, AbFrontier), PDIA3 (1:400 dilution, AbFrontier), and PRDX2 (1:200 dilution, AbFrontier). The sections were incubated in primary antibody solution for 1 h at room temperature. All samples were incubated for 30 min with universal biotinylated secondary antibodies at room temperature.
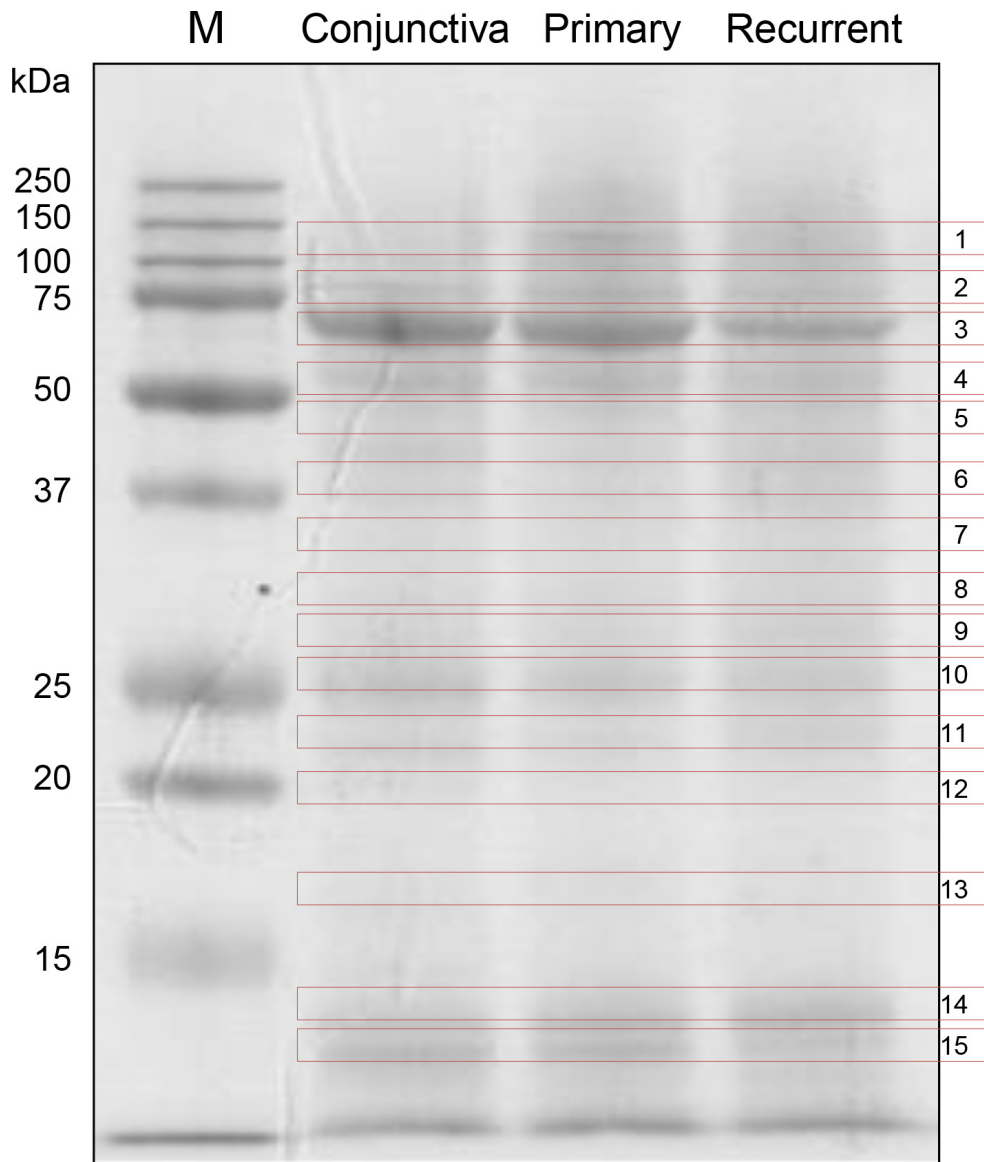
Figure 1. Identification of differentially expressed proteins in pterygium compared to healthy conjunctiva. Crude extracts of healthy conjunctiva as well as primary and recurrent pterygial tissues of eight subjects from each group were pooled; 50 μg of protein from each pooled sample was separated with SDS–PAGE and stained with Coomassie brilliant blue. Fifteen bands of each sample were excised and subjected to in-gel digestion. Digested peptides were then fractionated and identified with LC-MS/MS analysis. Molecular weight markers are shown in the left side of the figure.

Samples were then washed twice, and final incubation was performed for 30 min using streptavidin-peroxidase. Signals were developed using 3,3-diaminobenzidine for 5 min and counterstained with Mayer's hematoxylin (Dako, Glostrup, Denmark). Negative controls were prepared by leaving out the primary antibody.

RESULTS

Protein Identification with LC-ESI-MS/MS analysis: Pooled crude extracts of eight individuals from each group (normal conjunctiva, pterygium, and recurrent pterygia) were run on an SDS–PAGE gel, and the gel was stained with Coomassie brilliant blue G-250 (Figure 1). The stained SDS–PAGE gel was cut into 15 pieces according to molecular weight and

subjected to in-gel digestion. Digested peptides were then fractionated and identified with LC-MS/MS. All the experiments were replicated twice. In the first experiment, 346 proteins were differentially expressed in the three groups. Among these, 249 protein expression patterns were replicated in the second experiment.

First, we analyzed the pterygia-specific proteins by comparing normal conjunctiva with primary and recurrent pterygium. The expression of 153 proteins was downregulated (Appendix 1), and 77 proteins were upregulated in pterygia (Appendix 2).

Ontology analysis of pterygium-specific proteins: Using a web-based gene ontology program, DAVID, 230 proteins that

were up- or downregulated in pterygia tissues were classified. Molecular functions were attributed to 230 proteins using GOTERM_MF_FAT from the DAVID database. The related gene ontology categories were merged into a cluster using the DAVID functional clustering module. The major relevant categories were as follows: structural molecule activity, nucleotide binding, cytoskeletal protein binding, peptidase activity, guanyl ribonucleotide binding, structural constituent of eye, and oxidoreductase activity (Table 1). Interestingly, a group of proteins closely related to the structural molecule and peptidase activity were either up- or downregulated in pterygium. These data suggest that tissue rearrangement is strongly involved in pterygia formation and growth.

Selection of proteins for validation: The number of different proteins in the pterygium tissues was still too high to be validated. To narrow the possible candidate proteins, we further analyzed the proteomics data. Instead of comparing two groups (normal conjunctiva and pterygia), we compared proteome from three groups: normal conjunctiva, primary pterygium, and recurrent pterygium. Several fundamental profile patterns could be identified from these analyses, and we focused on increasing or decreasing patterns: upregulated in primary pterygium and further increased in recurrent pterygium or downregulated in primary pterygium and further decreased in recurrent pterygium. We thought that if the change in expression remained or increased in recurrent pterygium, the molecule should be considered to have a critical role in pterygia formation or growth. In this context, 40 proteins were differentially expressed in primary and recurrent pterygia. Among them, we chose three proteins, ALDH3A1, PDIA3, and PRDX2, for further validation. As shown in Table 2, the expression of all three proteins was significantly increased in pterygium and further increased in recurrent pterygium. Interestingly, all three proteins have some roles related to oxidative stress. PDIA3 and ALDH3A1 were classified into cell redox homeostasis (GO:0045454) and oxidation-reduction process (GO:0055114) groups, respectively. PRDX2 was also associated with cellular response to oxidative stress (GO:0034599).

TABLE 1. DAVID ANALYSIS RESULTS FOR SIGNIFICANTLY UP- OR DOWN- REGULATED PROTEINS IN PTERYGIUM TISSUES.

Term	Count	%	P-Value	Benjamini
Upregulated proteins				
structural molecule activity	32	21.9	7.60E-14	2.20E-11
nucleotide binding	32	21.9	3.10E-02	4.00E-01
cytoskeletal protein binding	17	11.6	3.90E-05	1.90E-03
peptidase activity	11	7.5	6.00E-02	5.30E-01
guanyl ribonucleotide binding	10	6.8	1.40E-02	2.40E-01
structural constituent of eye lens	6	4.1	5.00E-07	3.70E-05
glycosaminoglycan binding	6	4.1	1.30E-02	2.50E-01
phosphotransferase activity	3	2.1	4.20E-02	4.50E-01
Downregulated proteins				
structural molecule activity	12	17.4	1.60E-04	1.70E-02
peptidase activity	7	10.1	4.30E-02	4.70E-01
identical protein binding	7	10.1	7.90E-02	6.50E-01
carbohydrate binding	6	8.7	2.50E-02	3.70E-01
protein binding, bridging	5	7.2	9.80E-04	6.80E-02
glycosaminoglycan binding	5	7.2	4.20E-03	1.40E-01
polysaccharide binding	5	7.2	5.90E-03	1.70E-01
oxidoreductase activity, acting on peroxide as acceptor	3	4.3	9.80E-03	2.10E-01
antioxidant activity	3	4.3	2.00E-02	3.30E-01
integrin binding	3	4.3	3.10E-02	3.90E-01
growth factor binding	3	4.3	8.70E-02	6.60E-01

The Categories "GO-Molecular Function" were selected for testing. Term, specific terms within DAVID-categories; p value, p values for enrichment calculated by the EASE method used in DAVID; Benjamini, p values corrected for multiple testing by the Benjamini Hochberg method.

TABLE 2. PROTEINS IDENTIFIED BY LC-MS/MS ANALYSIS IN NORMAL CONJUNCTIVA, PRIMARY AND RECURRENT PTERYGIUM TISSUE.

Identified Proteins	Accession number	Peptide Hits		
		Normal conjunctiva	Primary pterygium	Recurrent Pterygium
ALDH3A1 Aldehyde dehydrogenase, dimeric NADP-preferring	IPI00296183	5	11	17
ATP5B ATP synthase subunit beta, mitochondrial	IPI00303476	0	2	3
COL1A1 Collagen alpha-1(I) chain	IPI00297646	3	7	9
COL1A2 Collagen alpha-2(I) chain	IPI00304962	0	20	23
EIF3H Eukaryotic translation initiation factor 3, isoform CRA_b	IPI00647650	0	3	9
FBLN5 Fibulin-5	IPI00294615	6	12	68
FH Isoform Mitochondrial of Fumarate hydratase, mitochondrial	IPI00296053	26	53	254
IGHV4–31; Putative uncharacterized protein	IPI00384938	0	8	36
IGKC Ig kappa chain C region	IPI00940069	0	10	14
MANF mesencephalic astrocyte-derived neurotrophic factor	IPI00328748	0	8	54
OBSCN Isoform 1 of Obscurin	IPI00288940	69	99	141
POSTN Periostin, osteoblast specific factor	IPI00410241	0	0	13
PRDX2 Peroxiredoxin-2	IPI00027350	5	12	13
RPSAP15;RPSA 33 kDa protein	IPI00413108	0	9	11
SLC4A1solutecarrierfamily4	IPI00791534	0	6	7
TACSTD2 Tumor-associated calcium signal transducer 2	IPI00297910	5	12	34
TGM2 Isoform 1 of Protein-glutamine gamma-glutamyltransferase 2	IPI00294578	11	21	39
TYMP Thymidine phosphorylase	IPI00292858	7	12	26
PDIA3 Protein disulfide-isomerase A3	IPI00025252	0	9	10
APEX1 DNA-(apurinic or apyrimidinic site) lyase	IPI00215911	7	2	0
ATP5J2 Putative uncharacterized protein ATP5J2	IPI00219291	12	9	3
CALML3 Calmodulin-like protein 3	IPI00216984	13	8	3
CLC Eosinophil lysophospholipase	IPI00216071	6	3	0
CRYBB2 Beta-crystallin B2	IPI00218748	42	29	9
GOT1 Aspartate aminotransferase, cytoplasmic	IPI00219029	7	5	0
HIST1H2AC Histone H2A type 1-C	IPI00216456	36	21	4
HPRT1 Hypoxanthine-guanine phosphoribosyltransferase	IPI00218493	6	4	2
IGJ Immunoglobulin J chain	IPI00178926	37	3	0
LMNB1 Lamin-B1	IPI00217975	12	8	0
NCL NCL protein	IPI00183526	8	2	0
PLS3 Plastin-3	IPI00216694	10	6	3

Identified Proteins	Accession number	Peptide Hits		
		Normal conjunctiva	Primary pterygium	Recurrent Pterygium
PSMA2 Proteasome subunit alpha type-2	IPI00219622	150	117	58
RPL22 60S ribosomal protein L22	IPI00219153	4	3	0
RPL30 60S ribosomal protein L30	IPI00219156	4	3	0
STOM Erythrocyte band 7 integral membrane protein	IPI00219682	5	4	0
TAGLN Transgelin	IPI00216138	14	8	3
TTN Isoform 7 of Titin	IPI00179357	18	3	0

Western blot analysis and immunohistochemistry: To validate the proteomic analysis data, we performed a western blot analysis of homogenates from normal conjunctiva and pterygium (100 μg of protein/lane). Our data showed that expression of the ALDH3A1, PDIA3, and PRDX2 proteins was significantly increased in the pterygia tissue compared to normal conjunctiva. This finding was consistent with the proteome analysis data (Figure 2). Using immunohistochemistry, upregulation of ALDH3A1, PDIA3, and PRDX2 expression in pterygia was also detected. As shown in Figure 3, all three proteins were mainly detected in the epithelial layer, and their intensities were increased in pterygium tissues compared to normal conjunctiva. Interestingly, these proteins were preferentially localized to the basal layer of the pterygial epithelium. These results confirmed that these three proteins were expressed at lower levels in normal conjunctiva and were present at considerably higher levels in the pterygium tissue.

DISCUSSION

The mechanism of pterygia formation is not yet fully understood, and its origin remains controversial. Despite many publications showing the influence of UV radiation in the pathogenesis, the role of oxidative stress and the antioxidant system is still unclear. The presence of oxidative stress in pterygium has been proved by finding an increase in 8-hydroxydeoxyguanosine (8-OHdG), malonedialdehyde (MDA), and decreased activity of superoxide dismutase, catalase, and glutathione peroxidase [7,23].

In this study, a proteomic approach was used to identify putative differences in pterygium compared to healthy conjunctiva. The expression of 153 proteins was downregulated (Appendix 1), and 77 proteins were upregulated in pterygia (Appendix 2). Our results revealed that proteins associated with an increase in extracellular matrix, structural, and mitotic proteins and those involved in tissue invasion were significantly affected. A remarkable change was also found in the group of proteins associated with oxidative stresses and protection against them. Among them, we confirmed increased expression of ALDH3A1, PDIA3, and *PRDX2* in pterygial epithelium.

ALDH3A1 is an abundant corneal crystalline in mammals, and it has been known to contribute to corneal defense by playing a multifunctional role in the protection of the cornea, and perhaps the entire eye, against UV-induced oxidative stress [24,25]. ALDH3A1 is a member of the ALDH superfamily of proteins that catalyze the NAD(P)-dependent oxidation of a wide range of endogenous and exogenous aldehydes. The central roles and mechanisms by which ALDH3A1 protects against UV-induced damage are thought to include the following: 1) metabolism of the toxic aldehydes, 2) direct absorption of UV light, 3) antioxidant function either directly through the scavenging of free radicals or indirectly
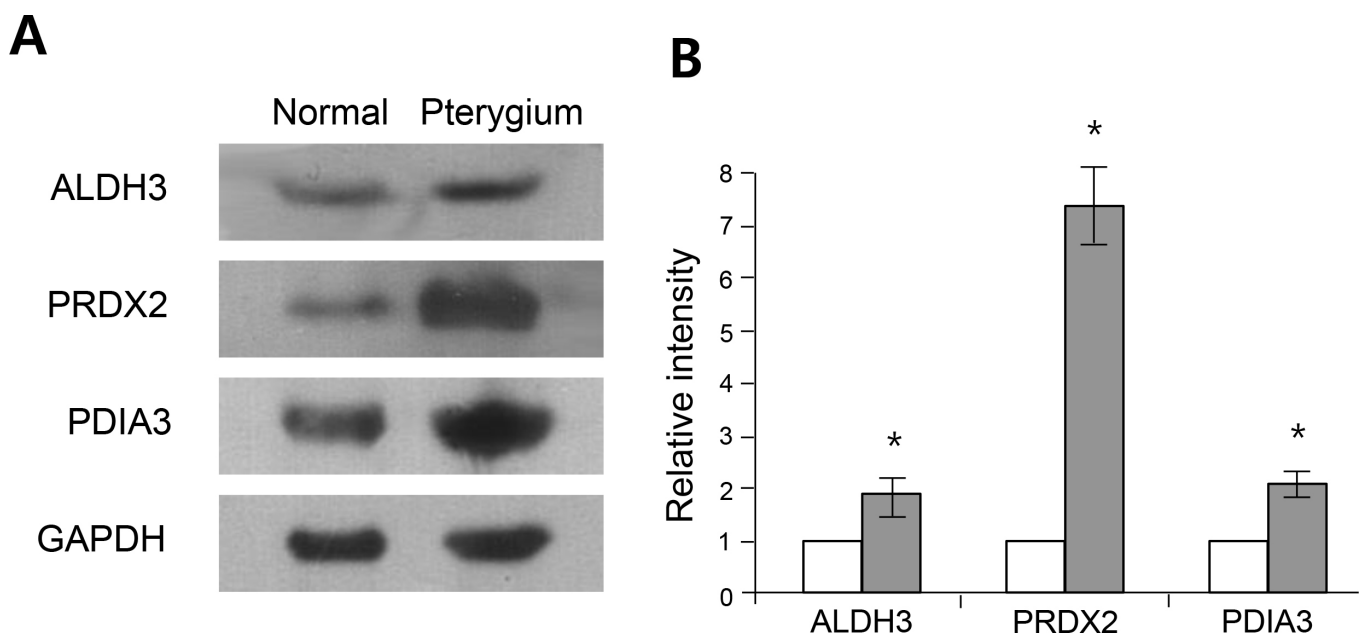

Figure 2. Western blot analysis of ALDH3A1, PDIA3, and PRDX2. **A**: Representative images of western blot analysis, examining the expression of ALDH3A1, PDIA3, and PRDX2 in pterygium and healthy conjunctiva tissue. GAPDH protein was used as an internal control. **B**: Statistically significant differences were observed between the pterygium and the healthy conjunctiva ($p<0.05$, n=6 in each group).
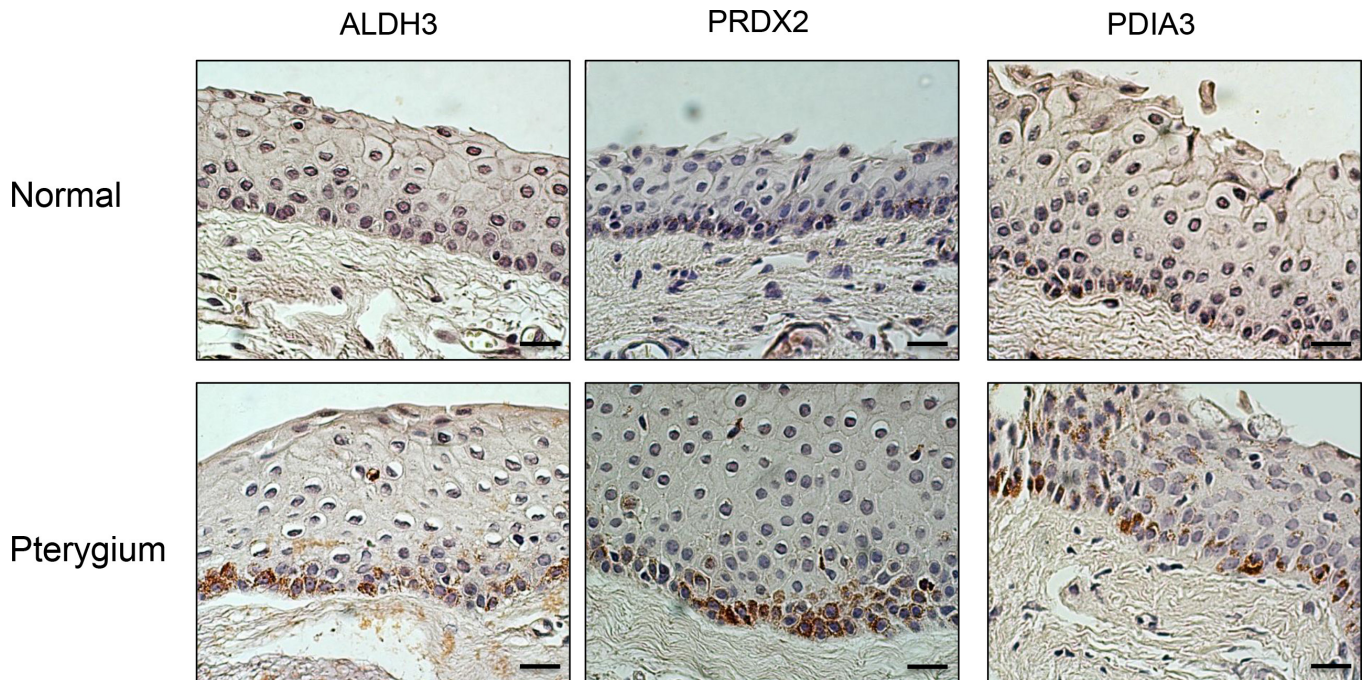
Figure 3. Immunolocalization of ALDH3A1, PDIA3, and PRDX2 in pterygium. Immunohistochemistry assay showed the presence of ALDH3A1, PDIA3, and PRDX2 preferentially in the cytoplasm of the basal epithelium of the pterygium. Meanwhile, there was only faintly positive immunostaining of these proteins in the healthy conjunctiva.

through the production of NADPH, 4) maintaining corneal refractive and transparent properties as a corneal crystalline, 5) chaperon-like activity, and 6) cell cycle regulation [25-28]. DNA is one of the major targets of UV-induced oxidative stress, and modified DNA, if left unchecked, may result in genetic mutation or cell death. Cell cycle control by ALDH3A1 resonates with the antiapoptotic and cell growth regulating roles of the lens alpha-crystallins [29,30]. Despite the upregulated expression of ALDH3A1 in pterygial tissue, we noted decreased expression of other crystalline proteins (alpha-crystallin A chain, alpha-crystallin B chain, beta-crystallin A3, beta-crystallin B1, beta-crystallin B2, and beta-crystallin S) in pterygia. This result suggests specific roles of ALDH3A1 in pterygia compared to other crystallins that have structural roles in conjunctiva.

PDIA3, another biomarker in our study, is a member of the endoplasmatic reticulum (ER) stress signaling pathway also known as unfolded protein response (UPR). The expression level of PDIA3 increases in response to cellular stress due to its function as a chaperone [31,32]. Recently published data connected PDIA3 to the apoptotic process and demonstrated PDIA3 had an antiapoptotic effect in the melanoma cell line A375 after ER stress was induced [33]. *PDIA3* possibly plays a role in the malignant transformation of prostate and cervical cancer [34,35]. The expression of this

gene is induced during neoplastic transformation possibly leading to redox-dependent modulation of cancer-relevant regulatory factors [36,37]. These previous findings suggest that the observed increase of PDIA3 in pterygium is most likely due to elevated cellular stress, which may be induced by oxidative damage. In addition to a role in the ER stress pathway, PDIA3 has recently gained attention due to its function as a component of the peptide-loading complex of the major histocompatibility complex (MHC) class I pathway [38]. In Pdia3-deficient mice, this complex is impaired and negatively influences presentation of antigenic peptides [39]. This may help tumors escape from immune surveillance with cytotoxic T cells. It has been proposed that pterygium and neoplasia have common features, raising the possibility that pterygium is a neoplastic-like growth disorder. Whether PDIA3 plays a similar role in pterygium as in tumor formation is still unclear.

We also reported increased expression of PRDX2 in pterygium. This result confirmed the recent findings of Bautista-de Lucio et al. [20]; they also conducted a proteomic approach to identify disease-specific protein expression and identified upregulated expression of PRDX2 in the pterygium. PRDX2 is a cytoplasmic enzyme that reduces intracellular reactive oxygen species (ROS) levels. It has been described that PRDX2 overexpression protects significantly

from peroxide-induced apoptosis and necrosis, while down-regulation of this enzyme promotes injurious effects of oxidative stress in cardiomyocytes [40]. A recent report found that pterygia express high levels of cellular proliferation proteins and low levels of apoptosis markers, suggesting that there is a disruption in the equilibrium among proliferation and apoptosis favoring cell proliferation [41]. The antiapoptotic role of this enzyme has been demonstrated in red blood cells and pancreatic cells as well [42,43]. Many isoforms of the peroxiredoxin family have been implicated in the survival of several types of ocular cells [44-47]. These data support the proposal that PRDX2 could be involved in inhibiting apoptosis in the pterygium.

Another important aspect of this study is the immuno-histochemical localization of ALDH3A1, PDIA3, and PRDX2 in pterygia. Interestingly, all three proteins are preferentially localized in the pterygium basal epithelium. Previous studies have reported that the expression of mutant p53 and other important apoptosis-related proteins, bax and bcl-2, was noted predominantly in the basal epithelial layer of pterygia [10,41]. Immunohistochemical studies demonstrated that apoptotic cells are found mainly confined to basal epithelial cells in pterygia [10,41], leading the chance that ALDH3A1, PDIA3, and PRDX2 are involved in regulating apoptosis and cell proliferation in pterygia. The antiapoptotic role of these three proteins has been demonstrated in various tissues [24,28,33,40,42,43]. Further studies are required to establish the functional roles of these proteins in pterygia formation.

Limited investigation regarding differences in the pathogenesis of primary and recurrent pterygia has been performed. We focused on proteins that show graded patterns of overexpression in primary and recurrent pterygia compared to normal conjunctiva. We presumed that these proteins might have more critical roles in pterygia formation and recurrence. We also focused on the difference in proteomic data between primary and recurrent pterygium, because the mechanisms that determine the genesis and the recurrence of a pterygia may not be the same. Interestingly, our result showed that periostin is a pathognomic marker for recurrent pterygium, and it was not expressed in primary pterygium or in normal conjunctiva. This finding confirms Kuo et al.'s transcriptome study [48,49]. It seemed reasonable to assume that recurrent pterygia possess molecular changes related to their recurrence.

In this study, we reported increased expression of ALDH3A1, PDIA3, and PRDX2 in pterygia using a proteomic approach. All three proteins are presumed to have significant roles related to oxidative stress. Our results are consistent with the hypothesis that oxidative stress is a significant factor in the pathogenesis of pterygia. We hypothesized that UV exposure and resultant oxidative stress leads to increased ALDH3A1, PDIA3, and PRDX2 levels, which provide resistance to UV-induced apoptosis and a hyperproliferative state.

APPENDIX 1.

Down-Regulated Proteins in pterygia, Identified by LC-MS/MS. To access the data, click or select the words "Appendix 1."

APPENDIX 2.

Up-Regulated Proteins in pterygia, Identified by LC-MS/MS. To access the data, click or select the words "Appendix 2."

REFERENCES

1. Solomon AS. Immunologic basis for the pathogenesis of pterygium. Am J Ophthalmol 1985; 99:216-7. [PMID: 3970133].

2. Hill JC, Maske R. Pathogenesis of pterygium. Eye (Lond) 1989; 3:218-26. [PMID: 2695353].

3. Chui J, Di Girolamo N, Wakefield D, Coroneo MT. The pathogenesis of pterygium: current concepts and their therapeutic implications. Ocul Surf 2008; 6:24-43. [PMID: 18264653].

4. Todani A, Melki SA. Pterygium: current concepts in pathogenesis and treatment. Int Ophthalmol Clin 2009; 49:21-30. [PMID: 19125061].

5. Saw SM, Tan D. Pterygium: prevalence, demography and risk factors. Ophthalmic Epidemiol 1999; 6:219-28. [PMID: 10487976].

6. Bradley JC, Yang W, Bradley RH, Reid TW, Schwab IR. The science of pterygia. Br J Ophthalmol 2010; 94:815-20. [PMID: 19515643].

7. Balci M, Sahin S, Mutlu FM, Yagci R, Karanci P, Yildiz M. Investigation of oxidative stress in pterygium tissue. Mol Vis 2011; 17:443-7. [PMID: 21321673].

8. Perra MT, Maxia C, Corbu A, Minerba L, Demurtas P, Colombari R, Murtas D, Bravo S, Piras F, Sirigu P. Oxidative stress in pterygium: relationship between p53 and 8-hydroxydeoxy-guanosine. Mol Vis 2006; 12:1136-42. [PMID: 17093398].

9. Seet LF, Tong L, Su R, Wong TT. Involvement of SPARC and MMP-3 in the pathogenesis of human pterygium. Invest Ophthalmol Vis Sci 2012; 53:587-95. [PMID: 22222271].

10. Tan DT, Tang WY, Liu YP, Goh HS, Smith DR. Apoptosis and apoptosis related gene expression in normal conjunctiva

and pterygium. Br J Ophthalmol 2000; 84:212-6. [PMID: 10655200].

11. Di Girolamo N, Kumar RK, Coroneo MT, Wakefield D. UVB-mediated induction of interleukin-6 and −8 in pterygia and cultured human pterygium epithelial cells. Invest Ophthalmol Vis Sci 2002; 43:3430-7. [PMID: 12407153].

12. Chiang CC, Cheng YW, Lin CL, Lee H, Tsai FJ, Tseng SH, Tsai YY. Cyclooxygenase 2 expression in pterygium. Mol Vis 2007; 13:635-8. [PMID: 17515883].

13. Beden U, Irkec M, Orhan D, Orhan M. The roles of T-lympho-cyte subpopulations (CD4 and CD8), intercellular adhesion molecule-1 (ICAM-1), HLA-DR receptor, and mast cells in etiopathogenesis of pterygium. Ocul Immunol Inflamm 2003; 11:115-22. [PMID: 14533030].

14. Nguyen CQ, Peck AB. Unraveling the pathophysiology of Sjogren syndrome-associated dry eye disease. Ocul Surf 2009; 7:11-27. [PMID: 19214349].

15. Soria J, Durán JA, Etxebarria J, Merayo J, González N, Reigada R, García I, Acera A, Suárez T. Tear proteome and protein network analyses reveal a novel pentamarker panel for tear film characterization in dry eye and meibomian gland dysfunction. J Proteomics 2013; 78:94-112. [PMID: 23201116].

16. Hains PG, Truscott RJ. Proteome analysis of human foetal, aged and advanced nuclear cataract lenses. Proteomics Clin Appl 2008; 2:1611-9. [PMID: 21136811].

17. Stastna M, Behrens A, McDonnell PJ, Van Eyk JE. Analysis of protein composition of rabbit aqueous humor following two different cataract surgery incision procedures using 2-DE and LC-MS/MS. Proteome Sci 2011; 9:8-[PMID: 21306621].

18. Joseph R, Srivastava OP, Pfister RR. Differential epithelial and stromal protein profiles in keratoconus and normal human corneas. Exp Eye Res 2011; 92:282-98. [PMID: 21281627].

19. Frost MR, Norton TT. Alterations in protein expression in tree shrew sclera during development of lens-induced myopia and recovery. Invest Ophthalmol Vis Sci 2012; 53:322-36. [PMID: 22039233].

20. Bautista-de Lucio VM, López-Espinosa NL, Robles-Contreras A, Pérez-Cano HJ, Mejía-López H, Mendoza G, Jiménez-Martínez MC, Garfias Y. Overexpression of peroxiredoxin 2 in pterygium. A proteomic approach. Exp Eye Res 2013; 110:70-5. [PMID: 23499777].

21. Sung HJ, Ahn JM, Yoon YH, Rhim TY, Park CS, Park JY, Lee SY, Kim JW, Cho JY. Identification and validation of SAA as a potential lung cancer biomarker and its involvement in metastatic pathogenesis of lung cancer. J Proteome Res 2011; 10:1383-95. [PMID: 21141971].

22. Heo SH, Lee SJ, Ryoo HM, Park JY, Cho JY. Identification of putative serum glycoprotein biomarkers for human lung adenocarcinoma by multilectin affinity chromatography and LC-MS/MS. Proteomics 2007; 7:4292-302. [PMID: 17963278].

23. Tsai YY, Cheng YW, Lee H, Tsai FJ, Tseng SH, Lin CL, Chang KC. Oxidative DNA damage in pterygium. Mol Vis 2005; 11:71-5. [PMID: 15692461].

24. Pappa A, Chen C, Koutalos Y, Townsend AJ, Vasiliou V. Aldh3a1 protects human corneal epithelial cells from ultra-violet- and 4-hydroxy-2-nonenal-induced oxidative damage. Free Radic Biol Med 2003; 34:1178-89. [PMID: 12706498].

25. Estey T, Cantore M, Weston PA, Carpenter JF, Petrash JM, Vasiliou V. Mechanisms involved in the protection of UV-induced protein inactivation by the corneal crystallin ALDH3A1. J Biol Chem 2007; 282:4382-92. [PMID: 17158879].

26. Estey T, Piatigorsky J, Lassen N, Vasiliou V. ALDH3A1: a corneal crystallin with diverse functions. Exp Eye Res 2007; 84:3-12. [PMID: 16797007].

27. Estey T, Chen Y, Carpenter JF, Vasiliou V. Structural and functional modifications of corneal crystallin ALDH3A1 by UVB light. PLoS ONE 2010; 5:e15218-[PMID: 21203538].

28. Black W, Chen Y, Matsumoto A, Thompson DC, Lassen N, Pappa A, Vasiliou V. Molecular mechanisms of ALDH3A1-mediated cellular protection against 4-hydroxy-2-nonenal. Free Radic Biol Med 2012; 52:1937-44. [PMID: 22406320].

29. Kamradt MC, Chen F, Sam S, Cryns VL. The small heat shock protein alpha B-crystallin negatively regulates apoptosis during myogenic differentiation by inhibiting caspase-3 acti-vation. J Biol Chem 2002; 277:38731-6. [PMID: 12140279].

30. Andley UP, Song Z, Wawrousek EF, Brady JP, Bassnett S, Fleming TP. Lens epithelial cells derived from alphaB-crystallin knockout mice demonstrate hyperproliferation and genomic instability. FASEB 2001; 15:221-9. [PMID: 11149910].

31. Ni M, Lee AS. ER chaperones in mammalian development and human diseases. FEBS Lett 2007; 581:3641-51. [PMID: 17481612].

32. Corazzari M, Lovat PE, Armstrong JL, Fimia GM, Hill DS, Birch-Machin M, Redfern CP, Piacentini M. Targeting homeostatic mechanisms of endoplasmic reticulum stress to increase susceptibility of cancer cells to fenretinide-induced apoptosis: the role of stress proteins ERdj5 and ERp57. Br J Cancer 2007; 96:1062-71. [PMID: 17353921].

33. Aureli C, Gaucci E, Arcangeli V, Grillo C, Eufemi M, Chichi-arelli S. ERp57/PDIA3 binds specific DNA fragments in a melanoma cell line. Gene 2013; 524:390-5. [PMID: 23587917].

34. Pressinotti NC, Klocker H, Schäfer G, Luu VD, Ruschhaupt M, Kuner R, Steiner E, Poustka A, Bartsch G, Sültmann H. Differential expression of apoptotic genes PDIA3 and MAP3K5 distinguishes between low- and high-risk prostate cancer. Mol Cancer 2009; 8:130-[PMID: 20035634].

35. De Marco F, Bucaj E, Foppoli C, Fiorini A, Blarzino C, Filipi K, Giorgi A, Schininà ME, Di Domenico F, Coccia R, Butterfield DA, Perluigi M. Oxidative stress in HPV-driven viral carcinogenesis: redox proteomics analysis of

HPV-16 dysplastic and neoplastic tissues. PLoS ONE 2012; 7:e34366-[PMID: 22470562].

36. Cicchillitti L, Di Michele M, Urbani A, Ferlini C, Donat MB, Scambia G, Rotilio D. Comparative proteomic analysis of paclitaxel sensitive A2780 epithelial ovarian cancer cell line and its resistant counterpart A2780TC1 by 2D-DIGE: the role of ERp57. J Proteome Res 2009; 8:1902-12. [PMID: 19714814].

37. Coe H, Michalak M. ERp57, a multifunctional endoplasmic reticulum resident oxidoreductase. Int J Biochem Cell Biol 2010; 42:796-9. [PMID: 20079872].

38. Garbi N, Hammerling G, Tanaka S. Interaction of ERp57 and tapasin in the generation of MHC class I-peptide complexes. Curr Opin Immunol 2007; 19:99-105. [PMID: 17150345].

39. Garbi N, Tanaka S, Momburg F, Hammerling GJ. Impaired assembly of the major histocompatibility complex class I peptide-loading complex in mice deficient in the oxidoreductase ERp57. Nat Immunol 2006; 7:93-102. [PMID: 16311600].

40. Zhao W, Fan GC, Zhang ZG, Bandyopadhyay A, Zhou X, Kranias EG. Protection of peroxiredoxin II on oxidative stress-induced cardiomyocyte death and apoptosis. Basic Res Cardiol 2009; 104:377-89. [PMID: 19030911].

41. Liang K, Jiang Z, Ding BQ, Cheng P, Huang DK, Tao LM. Expression of cell proliferation and apoptosis biomarkers in pterygia and normal conjunctiva. Mol Vis 2011; 17:1687-93. [PMID: 21738398].

42. Nagababu E, Mohanty JG, Friedman JS, Rifkind JM. Role of peroxiredoxin-2 in protecting RBCs from hydrogen peroxide-induced oxidative stress. Free Radic Res 2013; 47:164-71. [PMID: 23215741].

43. Zhao F, Wang Q. The protective effect of peroxiredoxin II on oxidative stress induced apoptosis in pancreatic beta-cells. Cell and Bioscience. 2012; 2:22-[PMID: 22709359].

44. Fatma N, Kubo E, Sen M, Agarwal N, Thoreson WB, Camras CB, Singh DP. Peroxiredoxin 6 delivery attenuates TNF-alpha-and glutamate-induced retinal ganglion cell death by limiting ROS levels and maintaining Ca2+ homeostasis. Brain Res 2008; 1233:63-78. [PMID: 18694738].

45. Fatma N, Kubo E, Toris CB, Stamer WD, Camras CB, Singh DP. PRDX6 attenuates oxidative stress- and TGFbeta-induced abnormalities of human trabecular meshwork cells. Free Radic Res 2009; 43:783-95. [PMID: 19572226].

46. Tulsawani R, Kelly LS, Fatma N, Chhunchha B, Kubo E, Kumar A, Singh DP. Neuroprotective effect of peroxiredoxin 6 against hypoxia-induced retinal ganglion cell damage. BMC Neurosci 2010; 11:125-[PMID: 20923568].

47. Chhunchha B, Fatma N, Bhargavan B, Kubo E, Kumar A, Singh DP. Specificity protein, Sp1-mediated increased expression of Prdx6 as a curcumin-induced antioxidant defense in lens epithelial cells against oxidative stress. Cell Death Dis 2011; 2:e234-[PMID: 22113199].

48. . Kuo CH. Miyazaki D, Nawata N, Tominaga T, Yamasaki A, Sasaki Y, Inoue Y. Prognosis-determinant candidate genes identified by whole genome scanning in eyes with pterygia. Invest Ophthalmol Vis Sci 2007; 48:3566-75. [PMID: 17652725].

49. Kuo CH, Miyazaki D, Yakura K, Araki-Sasaki K, Inoue Y. Role of periostin and interleukin-4 in recurrence of pterygia. Invest Ophthalmol Vis Sci 2010; 51:139-43. [PMID: 19661231].