Elevation of Human α-Defensins and S100 Calcium-Binding Proteins A8 and A9 in Tear Fluid of Patients with Pterygium

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Purpose. The pathogenesis of pterygia is still not well understood. Recent studies suggest that it may be associated with inflammation and progressive proliferation triggered by ultraviolet radiation. In this study the authors determined that the inflammatory nature of pterygium is reflected in the protein components of tears.

Methods. Consent for this study was obtained from 12 patients (average age, 57 years; eight men, four women) with unilateral pterygium. Tears were collected from diseased eyes and contralateral healthy control eyes with the use of fire-polished 10-μL calibrated glass pipettes before pterygium surgery. Tear protein profiles obtained from diseased and control eyes (seven patient samples) were compared using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) technology. Tears from another five patients with pterygium were used for subsequent protein identification experiments with nano-LC coupled with nanoelectrospray tandem mass spectrometry (nano-ESI-MS/MS).

Results. SELDI mass spectra showed that one peptide cluster with a molecular weight of 3.4 kDa and two proteins with molecular weights of 10.8 kDa and 12.7 kDa were elevated in tears of eyes with pterygium. Proteins of interest were identified by nano-LC-ESI-MS/MS as human α-defensins (human neutrophil peptide [HNP]-1, HNP-2, HNP-3) and S100 calcium-binding proteins A8 and A9. Mean concentrations ($n = 7$) of α-defensins were 1.33 ± 0.47 μg/mL (HNP-1, $P < 0.015$) and 0.61 ± 0.23 μg/mL (HNP-2, $P < 0.012$) for pterygium eyes and 0.17 ± 0.12 μg/mL (HNP-1) and 0.02 ± 0.02 μg/mL (HNP-2) for fellow control eyes. Compared with tears from eyes without pterygium or other abnormalities, the level of S100 A8 increased 1.4- to 13.4-fold (average fold change, 4.5) and S100 A9 increased 1.5- to 4.0-fold (average fold change, 2.3) in 4 of 7 patients.

Conclusions. The upregulated expression of human α-defensins and S100 A8 and A9 in tear fluids of patients with pterygium indicates that they may be part of the response of the ocular surface to the formation of this fibrovascular tissue or the accompanying inflammation. They may also serve as a useful indicator for predicting recurrent pterygium. (*Invest Ophthalmol Vis Sci.* 2009;50:2077–2086) DOI:10.1167/iovs.08-2604

Pterygium is an encroachment of fibrovascular tissue from the bulbar conjunctiva onto the cornea.[1,2] The pathogenesis of pterygia is not well understood. However, various mechanisms have been suggested by previous studies, including degeneration,[3] oxidative stress,[4] antiapoptosis,[5] extracellular matrix remodeling,[6–8] immunologic mechanisms,[9,10] angiogenesis,[11] viral infections,[12,13] and genetic factors.[14,15] These mechanisms result in the development of chronic inflammation with fibrovascular proliferation.[2,16–18] The involvement of ultraviolet B (UVB) radiation is supported by molecular studies[19–21] and epidemiologic studies that show a strong positive link between pterygia and sunlight exposure.[22] UVB radiation may induce secretion by ocular surface epithelium of proinflammatory cytokines such as IL-1β and TNF-α into the tears bathing the mucosal surface of the eye. Surgical removal of pterygium tissue is the only treatment; however, this is often accompanied by high recurrence rates.[23,24]

Tears are essentially extracellular fluid for the cells of the ocular surface and an easily accessible source for examining molecular signals secreted onto the epithelial cells of the ocular surface.[25] Reports have been published regarding changes in tear function and dry eye in conjunction with pterygium.[26–28] However, no information is available regarding molecular changes in tear fluid from patients with pterygium.

With the recent advanced proteomic technology such as multidimensional nano-LC coupled with tandem mass spectrometry and SELDI-TOF-MS high-throughput biomarker technology (ProteinChip; Ciphergen Biosystems, Fremont, CA), it is feasible to capture tear protein profile changes associated with disease states. SELDI-TOF-MS technology has been demonstrated as a powerful and rapid method to profile tear proteins.[29–33]

In this study, changes in the levels of tear proteins in patients with pterygium are discovered by making use of rapid SELDI-TOF-MS analysis combined with tandem mass spectrometry identification of proteins. Human α-defensins and the calcium-binding proteins S100 A8 and A9, upregulated in tear fluid from pterygium eyes, are associated with wounding and inflammation.

Methods

Patients and Tear Collection

All patients received counseling, and the tear collection procedure was explained in the patient consent form. The procedure was approved by the Ethics Committee of the Singapore Eye Research Institute and adhered to the tenets of the Declaration of Helsinki. Tears were collected 1 to 3 days before surgery from 12 patients (average age, 57 years; eight men, four women) with unilateral pterygium. Tears from

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contralateral eyes with no sign of disease served as controls. Briefly, as previously, tear collection was by 10-μL pipettes with fire-polished tips.[29,34] The tip of the capillary was laid in the space between the globe and the lid, allowing tears to flow by capillary action into the pipette. Tears were spun at 8000 rpm to remove cells and were frozen at −80°C until analysis was initiated.

SELDI-TOF Analysis

NP20 (normal phase, hydrophilic surface; SiO_2) arrays (Ciphergen Biosystems, Fremont, CA) were used in this study. Two microliters of human tears were applied onto each array spot and allowed to air dry. After that, each target was washed three times with 5 μL deionized water and allowed to air dry. A saturated solution of 0.8 μL of the energy-absorbing molecule α-cyano-hydroxy cinnamic acid dissolved in 50% acetonitrile containing 0.5% trifluoroacetic acid (TFA) was added (2×) to the spot and allowed to dry. All arrays (ProteinChip; Ciphergen Biosystems) were analyzed in a reader (PBS-II; Ciphergen Biosystems) according to an automatic data collection protocol with the following acquisition settings: high mass, 20 kDa; digitizer rate, 250 MHz; laser intensity, 155; sensitivity, 10. The instrument was operated in positive ion mode with a source and detector voltage of 1.8 kV. For interpretation, data were analyzed with the biomarker technology (ProteinChip; Ciphergen Biosystems) software (version 3.0). Tear protein profiles from pterygium and healthy eyes were normalized by total ion current using a specific function (Biomarker Wizard; Ciphergen Biosystems) in biomarker technology (ProteinChip; Ciphergen Biosystems) software (version 3.0).

Protein Identification

Pooled human tear samples (from three eyes with pterygium before surgery) were separated using reverse-phase high-performance liquid chromatography (HPLC). The HPLC system used for protein purification was a separations module (Waters 2695; Waters Associates, Milford, MA) with an autosampler and a 996 photodiode array detector. The analytical column had a 5-μm particle size and 300- Å pore size, measured 150 × 3.9 mm (Delta PAK C18; Waters Associates), and had a flow rate of 0.2 mL/min. Mobile phase A was 0.02% TFA with 0.1% acetic acid in water, and mobile phase B was 0.02% TFA with 0.1% acetic acid in acetonitrile. A linear gradient from 20% B to 30% B was used in the first 10 minutes, followed by 30% B to 70% B in the next 60 minutes, and finally from 70% B to 90% B in the last 10 minutes, achieving an HPLC run of 80 minutes in total. The eluent was monitored at 210 nm, and 15 peaks were collected. These 15 fractions were then lyophilized individually.

Protein digestion was performed with a trypsin digestion kit (ProteoExtract-All-In-One Trypsin Digestion Kit; Calbiochem, Darmstadt, Germany). The digestion procedure was followed according to the manufacturer's guidelines. Briefly, 30 μL extraction buffer 1 from the kit was added to dissolve the lyophilized proteins of interest. After centrifugation at 10,000*g* for 15 minutes, a 25-μL aliquot of the supernatant was transferred to a new tube, and 25 μL digest buffer and 1 μL reducing agent were added. The sample was then incubated at 37°C for 15 minutes, and 1 μL blocking agent was added to the sample and incubated at room temperature for 15 minutes. Then 1 μL trypsin (1 μg/μL) was added to the sample and incubated at 37°C for 2 hours. The resultant mixture was lyophilized and reconstituted using 2% MeOH with 1% formic acid in water. Digests were injected onto a 300-μm internal diameter × 5 mm precolumn (C18 PepMap100, 5 μm, 100 Å; Dionex; LC Packings, Sunnyvale, CA) with the use of an automated nanoflow liquid chromatography system (UltiMate; Dionex, LC Packings) with a nanospray source coupled to a quadrupole time-of-flight tandem mass spectrometer (QSTAR XL; Applied Biosystems, Foster City, CA). After washing and desalting for 5 minutes in mobile phase A (0.1% formic acid in water) at a flow rate of 0.03 mL/min, the peptides were eluted with a linear gradient of 5% to 60% B (0.1% formic acid in acetonitrile) over 45 minutes at a flow rate of 0.3 μL/min on a 75-μm i.d. × 10-cm fused silica column (New Objective, Woburn, MA) self-packed with C18 materials (Luna 3u C18, 3 μm, 100 Å; Phenomenex, Torrance, CA). Mass spectra were obtained in the positive ion TOF mode, with a scan range from m/z 300 to 1200; the MS/MS scan range was m/z 100 to 2500. The nanospray tip potential used for the analyses was 2200 V.

Results

Tear samples of both eyes were collected from 12 patients before surgery (Table 1). Tear samples from seven patients with pterygium (diseased eyes and fellow control eyes) were used for tear protein profile analysis on a NP20 chip (hydrophilic; ProteinChip; Ciphergen Biosystems), as described in our previous paper.[29] Tears from five additional patients with pterygium were used for subsequent protein identification. A typical healthy human tear protein profile (mass range, 1500–50,000 Da) on a NP20 chip is shown in Figure 1. Typical abundant tear proteins such as lysozyme (14,690.7 Da), lipophilin (16,428.4 Da), and lipocalin (17,428.3 Da) were observed. Representative SELDI-TOF spectra (gel view; mass range, 3000~3600 Da) of tear protein profiles from three patients with pterygium are seen in Figure 2. Each tear sample was analyzed in duplicate. In this region, we found three proteins with molecular weights of 3371.0, 3442.2, and 3486.2 Da that were upregulated in the tears of affected eyes. In fact, these three proteins were identified in our previous study[29] as human α-defensins (human neutrophil peptide [HNP]-1, 3442.2 Da; HNP-2, 3371.0 Da; HNP-3,

Table 1. Summary of Patient Information and Detection of S100 A8 and A9 by SELDI

Patient Code	Age (y)	Sex	Left Eye	Right Eye	S100 A8 (fold change, pterygium/control)	S100 A9 (fold change, pterygium/control)
P019	74	M	Pterygium	Control	13.4	4.0
P011	40	F	Pterygium	Control	4.0	2.1
P018	66	M	Pterygium	Control	3.4	1.5
P021	48	M	Control	Pterygium	3.4	1.5
P035	64	M	Pterygium	Control	3.0	N
P023	53	F	Pterygium	Control	2.6	N
P045	75	M	Control	Pterygium	1.4	N
P003	45	M	Pterygium	Control	ND	ND
P006	36	M	Pterygium	Control	ND	ND
P008	54	F	Pterygium	Control	ND	ND
P027	73	F	Pterygium	Control	ND	ND
P111	52	M	Control	Pterygium	ND	ND

P011, P018, P019, P021, P023, P035, and P045 were used for tear protein profiling experiments. P003, P006, P008, P027, and P111 were used for protein identification experiments. ND, samples were used for protein identification; therefore, no quantitative data were available; N, no signal detected.

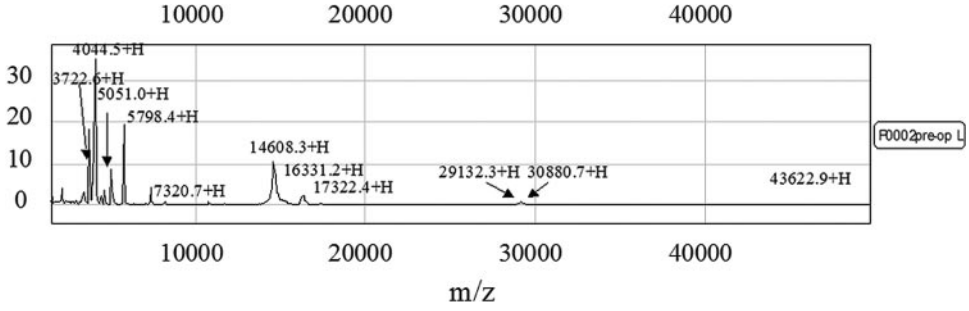


FIGURE 1. Typical human tear protein profile from a healthy eye observed with specialized technology (on a NP20 hydrophilic surface) in the range of 1500 Da to 50,000 Da.

3486.2 Da). In that study we also developed an LC-MS method for measuring the concentration of these antimicrobial peptides in human tears. The most abundant multiple charged ions, m/z = 861.5 ($[M+4H]^{4+}$) for HNP-1 and m/z = 844.0 ($[M+4H]^{4+}$) for HNP-2, were monitored simultaneously in selected-ion recording for quantification. Mean concentrations of α-defensins were 1.33 ± 0.47 μg/mL (HNP-1, $P < 0.015$) and 0.61 ± 0.23 μg/mL (HNP-2, $P <$

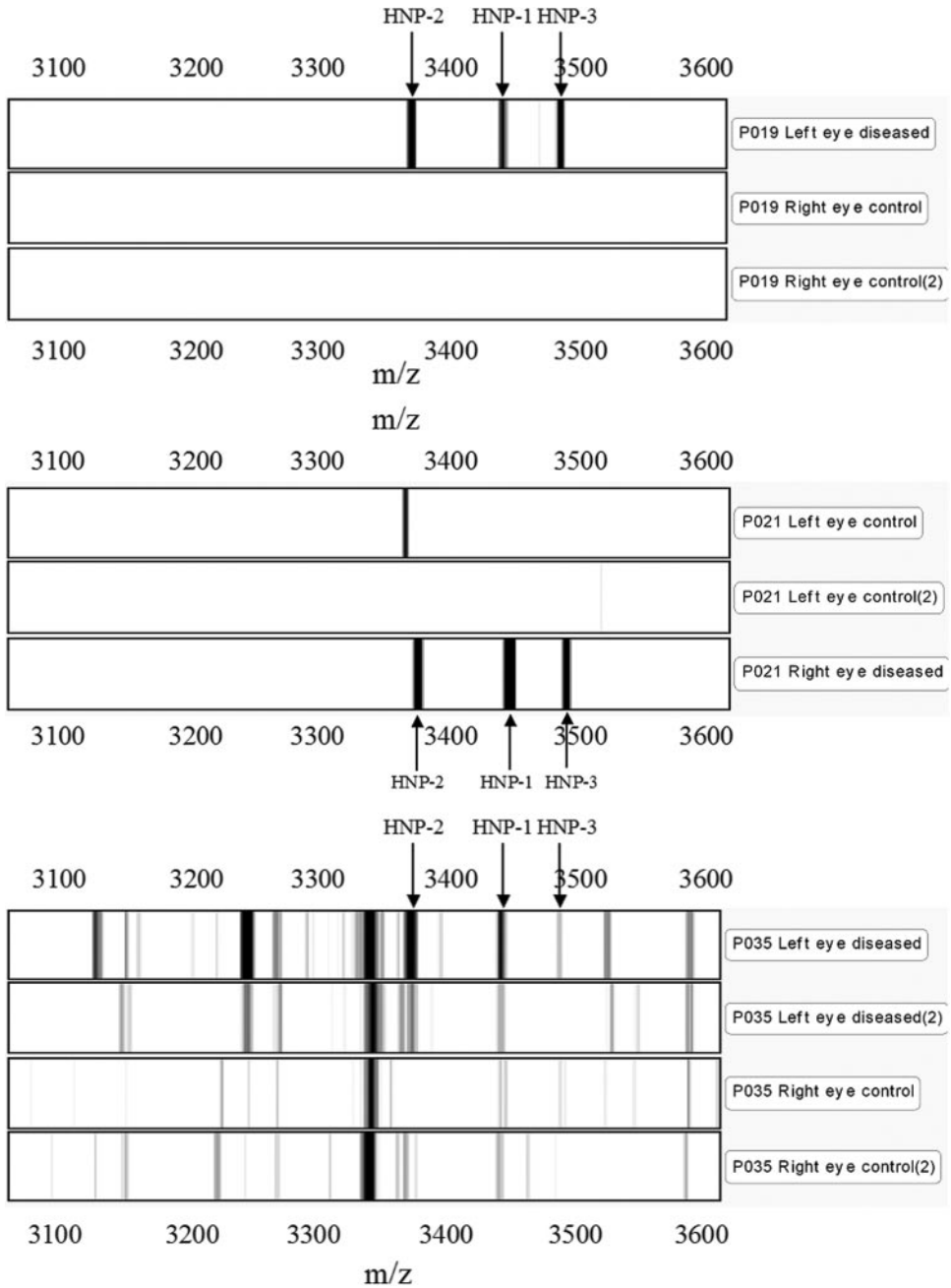


FIGURE 2. Three representative SELDI mass spectra (in gel view) showed the upregulated expression of three human α-defensins (HNP-1, HNP-2, HNP-3) in tear fluid from pterygium eyes.

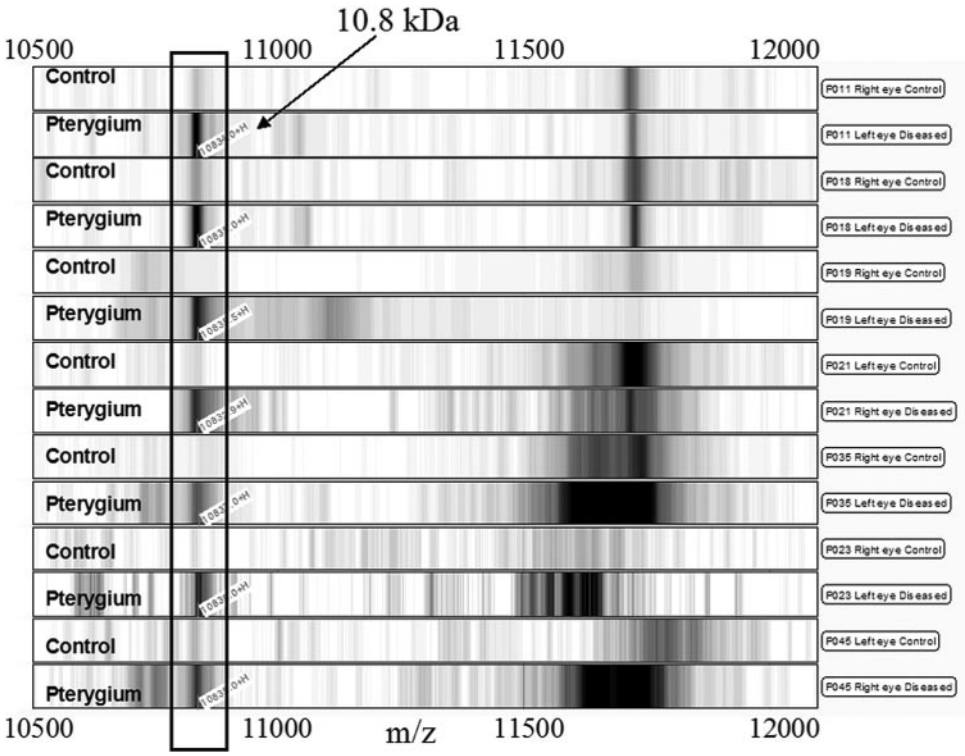


FIGURE 3. A series of SELDI mass spectra (in gel view) revealed that a protein with the molecular weight of 10.8 kDa was upregulated in tear fluids from pterygium eyes. Elevated levels of this protein were observed in 7 of 7 patients.

0.012) for eyes (n = 7) with pterygium and 0.17 ± 0.12 μg/mL (HNP-1) and 0.02 ± 0.02 μg/mL (HNP-2) for fellow control eyes, respectively.

Another clear change in the tears between pterygium eyes and control eyes was found in the mass range of 10,500 to 13,000 Da. Two proteins in this mass range (MWt 10.8 kDa and MWt 12.7 kDa) were elevated in tears from pterygium eyes (Figs. 3, 4). To identify these two proteins, tears pooled from five pterygium eyes were separated by reverse-phase HPLC (Fig. 5). In peak D, a 10.8-kDa protein at a retention time of 49.2 minutes and, in peak E, a 12.7-kDa protein with a retention time of 71.6 minutes were seen. Further LC-MS/MS analysis of tryptic digests of these two proteins indicated that the 10.8-kDa protein was S100 calcium-binding protein A8 and that the 12.7-kDa protein was S100 calcium-binding protein A9. In total, six peptide fragments originating from S100 A8 were observed. Two representative MS/MS spectra of two peptide fragments are seen in Figure 6. They are LLETEC*PQYIR (C* represents cysteine treated with iodoacetamide to form carbamidomethyl-cysteine), doubly charged at m/z = 711.26 (Mascot score = 100), and KGADVWFK, doubly charged at m/z = 475.70 (Mascot score = 59). For these two peptide fragments, all y-ions observed matched exactly with the expected peptide sequences. Another four peptide fragments (please refer to the Supplementary Material, online at http://www.iovs.org/cgi/content/full/50/5/2077/DC1, for MS/MS spectra) included ELDINTDGAVNFQEFLILVIK (triply charged at m/z = 797.80, Mascot score = 84), ALNSIIDVYHK (doubly charged at m/z = 636.90, Mascot score = 78), GNFHAVYR (doubly charged at m/z = 482.30, Mascot score = 39), and MLTELEK (doubly charged at m/z = 432.30, Mascot score = 36). The overall sequence coverage for the identification of S100 A8 was 71%. We also observed six peptide fragments from S100 A9 (two representative MS/MS spectra are shown in Fig. 7; additional MS/MS spectra can be found in the Supplementary Material). These six peptide fragments are VIEHIMEDLDTNADK (doubly charged at m/z = 871.90, Mascot score = 104), LGHPDTLNQGEFK (doubly charged at m/z = 728.30, Mascot score = 80), QLSFEEFIMLMAR (doubly charged at m/z = 807.90, Mascot score = 76), NIETIINTFHQYSVK (triply charged at m/z = 603.00, Mascot score = 46), DLQNFLK (doubly charged at m/z = 439.30, Mascot score = 28), and LTWASHEK (doubly charged at m/z = 486.30, Mascot score = 27). Sequence coverage for the identification of S100 A9 was 62%.

To determine the relative levels of S100 A8 in tear fluid from diseased eyes and control eyes, the ratio (fold) of peak intensity between diseased eyes and control eyes was calculated. For S100 A8, fold changes comparing diseased eyes and the control eyes ranged from 1.4 to 13.4 (average fold change, 4.5; Table 1). For S100 A9, we only observed signals (Fig. 3) from 4 of 7 patients. The fold changes of S100 A9 ranged from 1.5 to 4.0, with an average of 2.3 (Table 1). S100 A9 was not detectable in three patients probably because of the relatively low concentration.

DISCUSSION

SELDI-TOF chip (ProteinChip; Ciphergen Biosystems) technology can be regarded as a modified version of MALDI-TOF because both use TOF mass spectrometry as a detector. Instead of using the usual MALDI stainless steel plate, surfaces are specially treated either chemically (e.g., hydrophilic, hydrophobic, ion-exchange) or biologically (e.g., antibody, receptor) in SELDI chips. With this configuration, proteins in the sample bind selectively on modified surfaces, and nonspecific binding proteins and other components such as salts and lipids are washed away. Therefore, different surfaces may generate different protein patterns for the same sample. An advantage of SELDI-TOF is that it requires only microliter sample sizes to profile an intact protein mixture. Based on our experience and other published work, SELDI-TOF is a suitable technique to profile peptides and small proteins within the mass range of 1500~30 kDa with high sensitivity. The signal intensity of each peak from a SELDI-TOF mass spectrum is directly associated with the amount of each protein component in the sample.

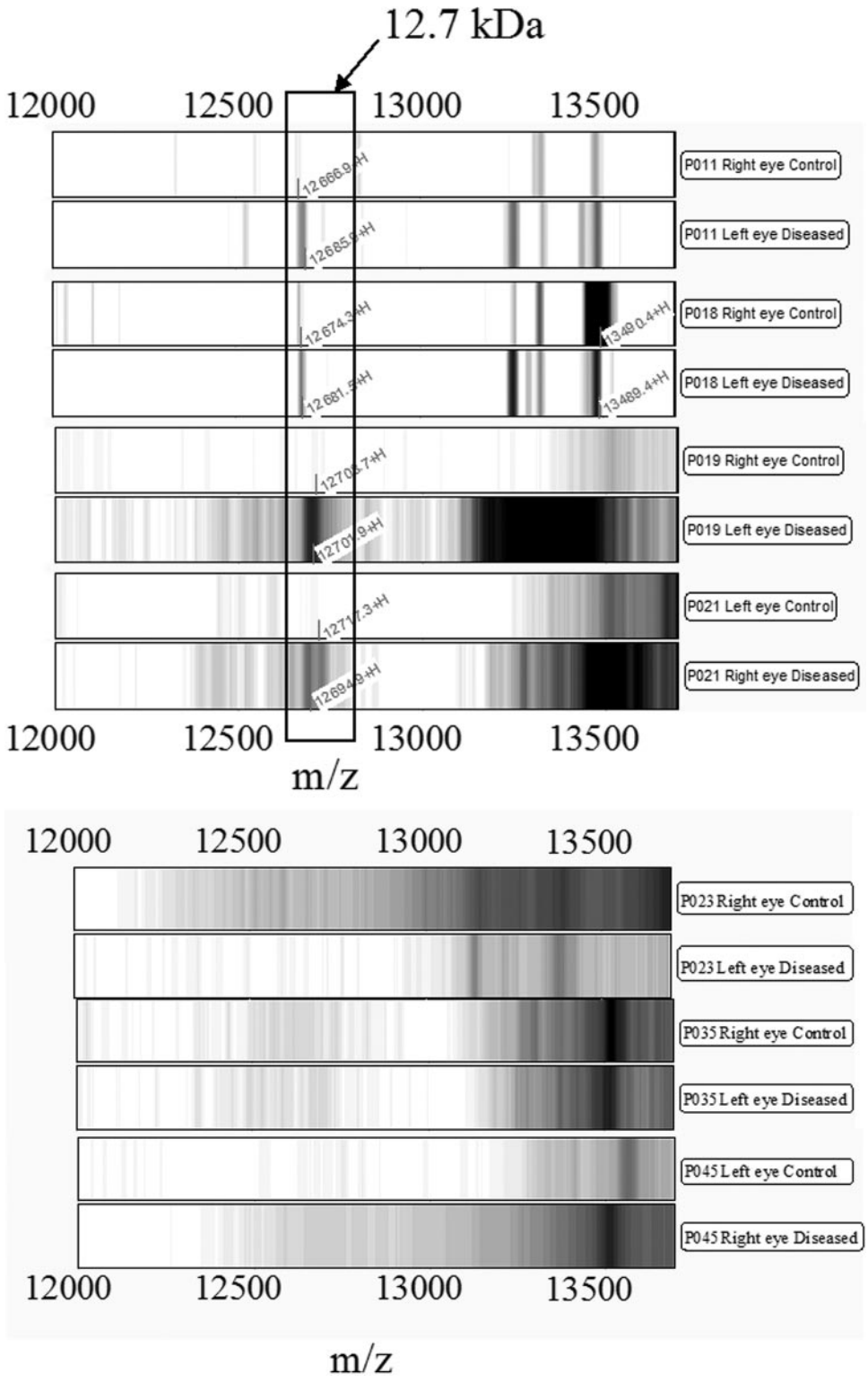


FIGURE 4. A series of SELDI mass spectra (in gel view) revealed a protein with the molecular weight of 12.7 kDa was upregulated in the tear fluid of pterygium eyes. However, the signal of this 12.7-kDa protein was observed in 4 of 7 patients.

SELDI is a high through-put technique used to generate protein profiles for initial screening and identifying of novel protein biomarkers. Application of this technology has shown much success in the early detection of ovarian and prostate cancers.[35,36] Compared with antibody-based techniques such as antibody array and Luminex (Austin, TX) multiplex bead assay, SELDI does not require prior knowledge of specific proteins or the use of antibodies. However, Luminex technology has higher sensitivity for a more limited number of cytokine targets.[37]

By combining our results[29] with those of Grus et al.,[31] 10 protein peaks can be comfortably assigned (Fig. 1) with SELDI for normal tears. Within this mass range, tear proteins, such as HNP-2 (3372.0 Da), HNP-1 (3448.0 Da), HNP-3 (3486.0 Da), nasopharyngeal carcinoma-associated proline-rich protein (4027 Da), proline-rich protein 4 (4052 Da), α-1-antitrypsin, C-terminal fragment

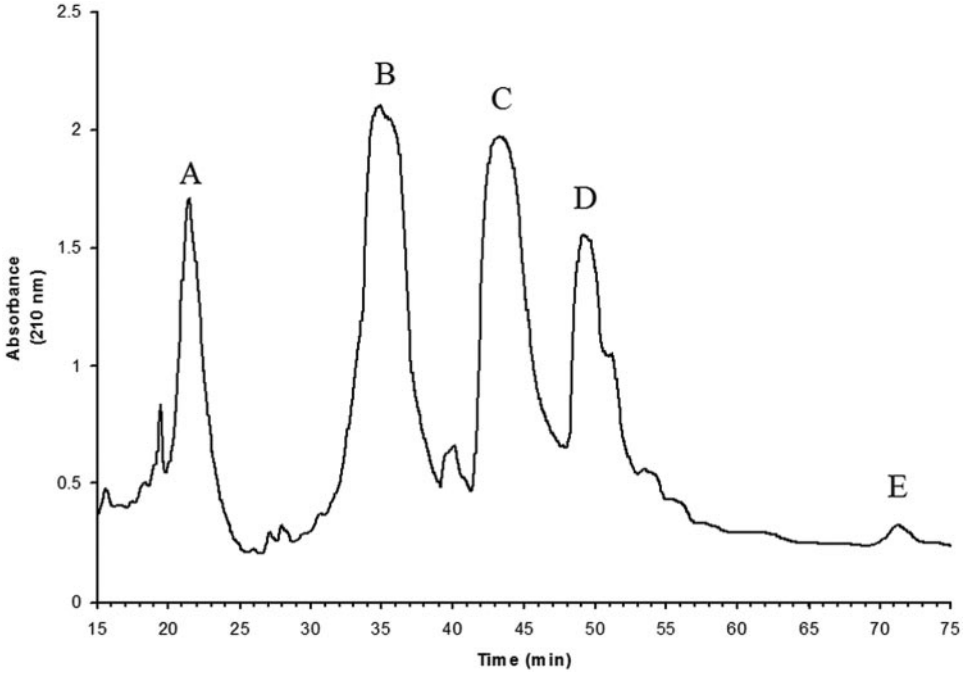


FIGURE 5. An HPLC chromatogram for separation and purification of these two proteins from the tears of eyes with pterygium. A 10.8-kDa protein was found in peak D at a retention time of 49.2 minutes, and a 12.7-kDa protein was observed in peak E at a retention time of 71.6 minutes.

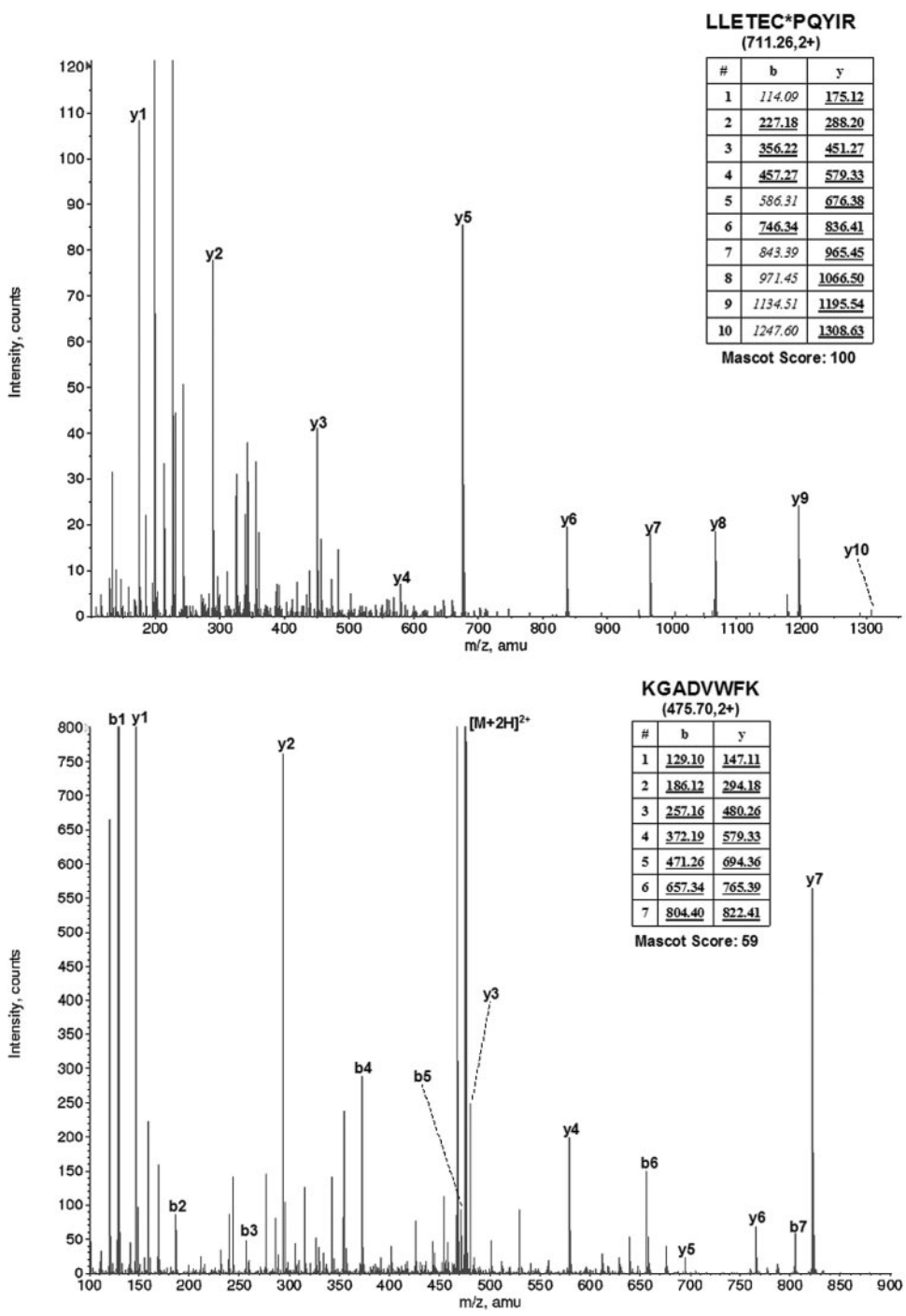
(4136 Da), proline-rich protein 3 (5792 Da), lysozyme (14,690.7 Da), lipophilin (16,428.4 Da), and lipocalin (17,428.3 Da) were observed. Other peaks are yet to be identified.

In this study, we found two obvious reproducible changes between pterygium eyes and healthy control eyes (one peptide cluster with a molecular weight around 3.4 kDa and two proteins with molecular weights of 10.8 kDa and 12.7 kDa). These peptides were subsequently identified as human α-defensins (HNP-1, HNP-2, HNP-3)[29] and S100 A8 and S100 A9 with high confidence using nano-LC-nano-ESI-MS/MS. Signal differences on the right-hand side of Figure 3 were inconsistent when we compared healthy with pterygium samples and probably were not disease related.

Defensins are small "cationic antimicrobial peptides" with approximately 29 to 45 amino acid residues and molecular weights of approximately 3 to 6 kDa.[38] Human defensins have six cysteine residues forming three intramolecular disulfide bonds. Among the six α-defensins found in humans and in nonhuman primates, human neutrophil peptides HNP-1 to HNP-4 are shown to be constituents of neutrophils and granulocytes. Another type of human defensin is β-defensin in which the alignment of the disulfide bridges is different from that of α-defensins.[38] Thus far, four β-defensins (hBD1-hBD4) have been identified, primarily in epithelial tissue. HNP-1 to HNP-3 can be found in the normal tear film and in inflamed conjunctival tissue.[39] The source of these three α-defensins is believed to be primarily from polymorphonuclear (PMN) cells residing on the ocular surface. In the case of inflammation or injury, the numbers of PMN cells increase dramatically. In acute conditions such as injury, the levels of α-defensins (released by PMN cells) can reach 10 μg/mL, which provides antimicrobial activity.[29] Like other mucosal surfaces, cornea and conjunctiva epithelial cells also express β-defensins. hBD-1 and hBD-3 are constitutively expressed in the epithelia of the ocular surface, whereas the expression of hBD-2 is inducible in infection and inflammation.[40,41] However, to the best of our knowledge, β-defensin proteins have not been observed in the tear film in healthy or diseased eyes.

In addition to direct antimicrobial properties, defensins are able to modulate other components of innate immunity.[42,43] Defensins act as chemoattractants for monocytes and neutrophils. Moreover, they influence the production of several cytokines. For example, in monocytes, TNF-α and IL-1β expression is stimulated by defensins. This suggests that the HNPs have the potential to modulate inflammatory responses through the regulation of cytokine production.[44] Proinflammatory cytokines, such as IL-1β and TNF-α, may be responsible for the increased expression of inhibiting matrix metalloproteinases (MMPs) in cultured pterygium fibroblasts, which in turn could result in extracellular matrix remodeling, angiogenesis, and fibroblast proliferation.[45] The source of α-defensins could be inflammatory cells, such as macrophages, lymphocytes on the ocular surface, and conjunctival epithelial cells.[33] α-Defensin levels probably reflect the inflammatory status of the ocular surface, an explanation that is also supported by a recent study from our laboratory.[29] Recently, increased amounts of α-defensins were found in the tears of patients with allergic conjunctival disease complicated by corneal lesions.[33] S100 A8 and A9 belong to the S100 calcium-binding protein family.[46] The S100 protein family is the largest group of calcium-binding proteins, and their biological functions are diverse and not yet fully defined. Human S100 A8 protein is also known as myeloid-related protein 8 (MRP-8) or calgranulin A, and S100 A9 is called MRP-14 or calgranulin B. S100 A8 and A9, with S100 A12, are major components of neutrophilic granulocytes. Growing evidence indicates that S100 A8, A9, and A12 form a new group of proinflammatory proteins.[46] Expression of the calcium-binding proteins is highly tissue specific. In other words, they are in circulating granulocytes and monocytes but not in resting tissue macrophages or lymphocytes. They are well known for their involvement in inflammatory disorders such as rheumatoid arthritis,[47,48] cystic fibrosis,[49] HIV infection,[50] and other autoinflammatory diseases. Recently, they have also been suggested as biomarkers for epithelial tumors.[51–59] For example, the overexpression of S100 A8 was observed in ovarian cancer,[51] prostate cancer,[55] and endometrial cancer.[54]

S100 A8 and S100 A9 usually form complexes, as suggested by recent studies.[46] Levels of S100 A8 and S100 A9 are elevated at sites of inflammation. S100 A8 protein is easily found and defined with SELDI. There are some difficulties observing S100 A9 and A12 signals by SELDI, probably because of signal sen-



#	b	y
1	*114.09*	175.12
2	227.18	288.20
3	356.22	451.27
4	457.27	579.33
5	*586.31*	676.38
6	746.34	836.41
7	*843.39*	965.45
8	*971.45*	1066.50
9	*1134.51*	1195.54
10	*1247.60*	1308.63

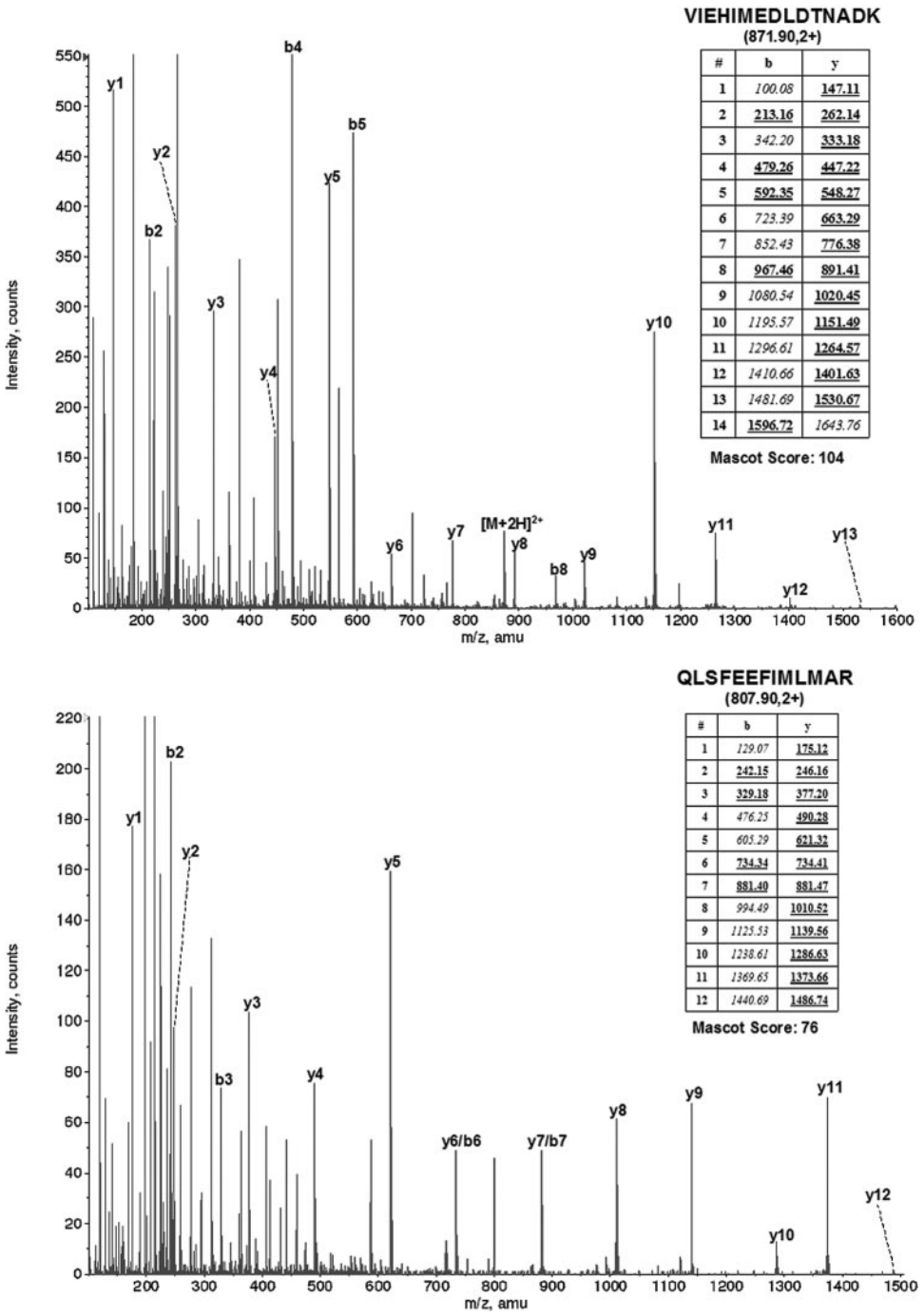
#	b	y
1	129.10	147.11
2	186.12	294.18
3	257.16	480.26
4	372.19	579.33
5	471.26	694.36
6	657.34	765.39
7	804.40	822.41

FIGURE 6. MS/MS spectra showed the fragment ions from one doubly charged precursor ion at m/z 711.26 (LLETEC*PQYIR; C* represents cysteine treated with iodoacetamide to form carbamidomethyl-cysteine) and another doubly charged precursor ion at m/z 475.70 (KGADVWFK), both originating from S100 calcium-binding protein A8. Peaks corresponding to *y* and *b* ions are marked.

sitivity. We observed S100 A9 (experimental average mass, 12,691 Da) from 4 of 7 pterygium eyes (Fig. 5; Table 1). The theoretical average mass of S100 A9 is 13,242 Da (from Swiss-Prot database). The 12.7-kDa signal we observed from the SELDI mass spectrum was actually a truncated form of S100 A9, which was previously reported by Vogl et al.[60] and Tolson et al.[61] The loss of the N-terminal MTCKM (−595 Da) and an additional acetylation (+42 Da) results in a difference of 553 Da between the intact form (13,242 Da) and the truncated form (theoretical average mass, 12,689 Da; our experimental average mass, 12,691 Da; error of 2 Da is acceptable for SELDI) of S100 A9.

Recent studies also show that S100 A8 and A9 can form a complex that has a cytotoxic effect on various tumor cell lines and normal fibroblasts by inducing apoptosis of these cells. Other functions of S100 A8 and A9 have been noted and include antimicrobial activity,[62] apoptosis,[63] inhibiting MMP activity,[64] and regulatory activities toward cells that participate in inflammation or immunologic reactions.

Our data showed overexpression of two inflammatory response proteins, S100 A8 and A9, in the tear fluid of patients with pterygium . This observation matches well with a recent microarray study on pterygium that showed a 2.2-fold increase for the gene expression of S100 A9 (calgranulin B).[65] Although the cause of pterygium is not clear, UV irradiation may be a key initial trigger for its pathogenesis. Overexpression of cytokines, growth factors, and perhaps S100 A8 and A9 can be induced by UV irradiation,[66] which further develops inflammation.



#	b	y
1	100.08	147.11
2	213.16	262.14
3	342.20	333.18
4	479.26	447.22
5	592.35	548.27
6	723.39	663.29
7	852.43	776.38
8	967.46	891.41
9	1080.54	1020.45
10	1195.57	1151.49
11	1296.61	1264.57
12	1410.66	1401.63
13	1481.69	1530.67
14	1596.72	1643.76

#	b	y
1	129.07	175.12
2	242.15	246.16
3	329.18	377.20
4	476.25	490.28
5	605.29	621.32
6	734.34	734.41
7	881.40	881.47
8	994.49	1010.52
9	1125.53	1139.56
10	1238.61	1286.63
11	1369.65	1373.66
12	1440.69	1486.74

FIGURE 7. MS/MS spectra showed the fragment ions from one doubly charged precursor ion at m/z 871.90 (VIEHIMEDLDTNADK) and another doubly charger precursor ion at m/z 807.90 (QLSFEEFIMLMAR), both originating from S100 calcium-binding protein A9. Peaks corresponding to *y* and *b* ions are marked.

Tear samples were collected at the late stage (i.e., a few days before surgery). Pterygia are typically accompanied by chronic inflammation. Compared with other chronic inflammatory ocular surface diseases such as dry eye (Beuerman RW, et al. *IOVS* 2006;47:ARVO E-Abstract 1940) and climatic droplet keratopathy (Beuerman RW, et al. *IOVS* 2007;48: ARVO E-abstract 1899), pterygium often showed higher S100A8/A9 levels in tears though S100A8/A9 is a general marker for inflammation. Proteomic analysis of tear proteins from these three ocular surface diseases also showed different protein profiles. α-Defensins and S100 A8 and A9 are upregulated in the tears of eyes with pterygium; both proteins are associated with different aspects of inflammation because they are correlated with the inflammatory state of the disease. Recent studies[47,48] show that S100 A8 and A9 proteins can be used as markers for the status of inflammation, and they may be useful for understanding the nature of pterygium. Under inflammatory conditions, a high concentration of S100 A8 and A9 may be toxic to ocular surface epithelial cells. A number of studies[5,67] have shown that apoptosis is part of pterygium and that the S100 A8/A9 proteins may have a role. This finding may lead to a new way to treat pterygium by suppressing the cytotoxic effect of S100 A8 and A9 on ocular surface tissue.[63]

In summary, the upregulated expression of human α-defensins and S100 A8 and A9 was found in tear fluids of pterygium eyes using SELDI chip (ProteinChip; Ciphergen Biosystems) technology. Human α-defensins and S100 A8 and A9

were identified by analyzing tryptic digests with nano-ESI-MS/MS. Overexpression of human α-defensins and S100 A8 and A9 in tear fluids may suggest their roles in the pathogenesis of the pterygium.

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