RESEARCH ARTICLE

Proteomic analysis of rabbit tear fluid: Defensin levels after an experimental corneal wound are correlated to wound closure

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The cornea is the major refracting optical element of the eye and therefore critical for forming a retinal image. The exposed surface of the eye is protected from pathogens by the innate immune system whose components include defensins, naturally occurring peptides with antimicrobial properties, and the physical barrier formed by the outer epithelial layer of the cornea. The proteomic approach has revealed that tear levels of defensins are correlated with the course of healing of an experimental corneal wound. Tears were collected from New Zealand White rabbits prior to (day 0) and daily for 5 days (days 1–5) following a standard unilateral 6 mm diameter corneal epithelial abrasion. Tear protein profiles obtained from wounded and contra-lateral control eyes were compared using SELDI ProteinChip technology. Peptides and proteins of interest were purified by RP-HPLC and characterized by nanoESI-MS/MS. Mass spectra of tears on post-wound day 1, revealed 13 peaks whose level decreased and five that increased. During wound healing the tear protein profile correlated with wound closure. An important finding was that the levels of rabbit defensins (NP-1 and NP-2), which were elevated after wounding returned to normal levels by the time the corneal abrasion healed. Relative quantification of NP-2 in tear fluid prior to (day 0) and after corneal wounding (days 1– 3) was determined using iTRAQ technology. A corneal wound eliminates the barrier function of innate immunity and puts the cornea at risk from microbial attack until the epithelial cells restore the surface barrier. The increased availability of defensins in the tears during healing suggests that these peptides could protect the cornea from microbial attack during a period of increased vulnerability.

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Abbreviations: EF, error factors; **IDA,** information-dependent acquisition; **MMTS,** methyl methanethiosulfonate; **PMN,** polymorphonuclear

1 Introduction

The cornea is the major refracting element in the light pathway forming the retinal image. Therefore, protection of the integrity of this transparent tissue is critical for good vision and survival of the organism. Mucosal surfaces are fluid environments as represented by the surface of the eye,

mouth, and airways which are protected from microbial invasion by mechanisms collectively referred to as innate immunity [1]. The innate immune system is comprised of both structural elements such as the barrier provided by an intact surface epithelium which limits pathogen entry and biochemical components such as the defensins, which have a cellular origin. Defensins, are small cationic peptides, and in the eye they are found in the tears where they can interact and potentially kill environmentally borne pathogens. A corneal wound breaches the important structural moiety of the innate immune system, but upregulation of the biochemical component, the defensins, may have a protective function at that time [1–3]. Other studies have shown that re-epithelialization of the wound is the first step to restoring the physical barrier component of innate immunity as well as the optical properties of the cornea and occurs in three phases [2, 3]: (i) the early neurogenic inflammatory phase which is characterized by reflex tearing, breakdown of the blood-ocular barrier, and release of polymorphonuclear leukocytes into the tears, and attachment sites between epithelial cells and basement membrane are broken; (ii) cell migration phase: corneal epithelial cells develop filopodia and lamellipodia and start to migrate across the stromal surface covering the wound area; (iii) cell proliferation phase: activation of limbal stem cells to completely regenerate the epithelial cellularity and organization.

After a breach of the epithelial surface, biogenic molecules in tear fluid become accessible to the extracellular space of the cornea. Copious tears are easily accessible for analysis of changes in peptide levels. Tears are a complex protein mixture and while changes in some peptides have been found, the fate of most of the more than one hundred tear proteins remains unknown during corneal wound healing. This is important as tear proteins have roles in regulating epithelial migration, proliferation and differentiation, cell-to-cell and cell-to-matrix interactions, stroma-extracellular matrix production, inflammation, scar formation, as well as protection against pathogens. Previous studies have identified some tear proteins that may participate in or mediate the corneal wound-healing cascade mentioned above. For example, fibronectin [4], a glycoprotein present in plasma and the extracellular matrix, has a role in epithelial cell migration and is a temporary substrate for cells migrating on the corneal surface. Other extracellular matrix related proteins released in tear fluids such as tenascin [5], plasmin, and plasminogen activator [6–8], and matrix metalloproteinases (MMP-8 and MMP-14) [9], also have a suggested role in the wound healing process. Neuropeptides such as substance P (SP) [10,11] and calcitonin gene-related peptide (CGRP) [12] are important molecules for their role in the neurogenic phase of a wound response, which results in the break-down of the blood-ocular barrier and access of large numbers of neutrophils into the tears [13]. A number of growth factors and cytokines including epidermal growth factor (EGF) [14–16], transformating growth factor (TGF-β1 and TGF-β2) [17], vascular endothelial growth factor (VEGF) [17], platelet-derived growth factor-BB (PDGF-BB) [18], hepatocyte growth factor (HGF) [19], basic fibroblast growth factor (bFGF) [20], connective tissue growth factor (CTGF) [21], tumour necrosis factor (TNFα) [22], and interleukins (IL-1 and IL-6) [23] have been found in tears and may modulate wound healing by stimulating epithelial growth, whereas others may trigger epithelial cell apoptosis.

Despite recent advances in understanding cellular behavior during corneal wound healing, much work remains to be done to identify tear borne cell-signaling components active in the wound-healing cascade. In an earlier study, the mass spectrum of rabbit tears several days after a corneal wound did reveal changes in a peak; however, the peptide components of that peak were not identified [24]. Previously it was found that the levels of human defensins were differentially changed after a surgical wound of the conjunctiva; however, due to the limitations in access to human tear samples, precise correlation with the healing process could not be made [25]. The rabbit model of corneal wound healing used here allows the wound healing process to be closely monitored so that the tear proteomics of wound healing can be easily correlated with healing. This study also shows that the upregulation of defensins occurs after a sterile wound to cornea.

SELDI-TOF-MS ProteinChip [26, 27] was used to screen for peptides related to the healing process. This technology utilizes affinity-modified surfaces to retain proteins based on their physical or chemical characteristics coupled with direct analysis by TOF-MS. Advantages of SELDI are the throughput and small sample volumes (2–3 μL). Previous work demonstrated that SELDI-TOF-MS ProteinChip technology is useful for profiling human tear proteins [25, 28–30]. This system has enabled detection of protein biomarkers directly from crude mixtures with minimal preprocessing. However, SELDI only provides mass/charge ratio (m/z), which makes it difficult to identify specific proteins of interest.

In the present study, levels of rabbit defensins were compared from tear protein profiles prior to (day 0) and after an experimental corneal wound (days 1–5) using SELDI-TOF-MS technology. Proteins/peptides of interest were subsequently purified using RP-HPLC and identified with nano-LC-nano-ESI-MS/MS. The quantitative results were independently verified using differential labeled tag technology (iTRAQ) [31–34].

2 Materials and methods

2.1 Chemicals

HPLC grade ACN and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid, TFA, acetic acid, ammonium bicarbonate, CHCA, DTT and iodoacetamide were purchased from Sigma (St. Louis, MO, USA). Trypsin was obtained from Roche Molecular Bio-

chemicals (Indianapolis, IN, USA). Water used in the mobile phase was either HPLC grade purchased from Fisher Scientific or Milli-Q grade (Millipore, MA, USA).

2.2 Experimental corneal wound

All animals were treated in accordance with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research. Tears from New Zealand White rabbits (2 kg, female, $n = 16$) were used for SELDI, HPLC purification, and protein identification experiments. An additional group of three rabbits was used for the iTRAQ experiment. The corneal wound (right eye only) was made as in a previous study by lightly impressing a 6 mm diameter trephine onto the corneal surface followed by removal of the epithelium with a spatula within the demarcated area to the level of the basal lamina [35]. The area of the wound was visualized by application of an innocuous disclosing dye, fluorescein, which is used in the ophthalmology clinic for wound disclosure on the ocular surface, in conjunction with a slit-lamp equipped with a cobalt-blue filter in the optical path for visualizing the stimulated light [35]. Measurements of the residual wound area were made over the time course of re-epithelialization [35].

2.3 Tear collection

Tears were collected from the inferior cul-de-sac using fire-polished 10 µL calibrated glass microcapillary tubes with care taken to avoid touching the ocular surface. Tears were collected 1 day prior to the wounding procedure (day 0) and at every day after wounding for 5 days (days 1–5) from both eyes (typically left eyes used as a control group and right eyes used as an experimental group). After collection, tear samples were immediately spun at 8000 rpm to remove cells and frozen at −80°C until analysis.

2.4 SELDI-TOF proteinChip

The normal-phase (NP-20) and RP (H4) ProteinChip arrays from Ciphergen Biosystems were used in this study. For analysis the RP surfaces of the ProteinChip arrays were pretreated with 30% ACN for 5 min. For detection, 2 µL of rabbit tears was applied onto each array spot and the array allowed to air dry. Each target was washed three times with 5 µL of 30% ACN containing 0.1% TFA for RP arrays and three washes of 5 µL of deionized water for normal-phase arrays prior to air drying. An energy absorbing molecule (EAM)-saturated CHCA dissolved in 50% ACN containing 0.5% TFA was added (0.8 µL, 2 ×) and allowed to dry. All ProteinChip Arrays were analyzed in a ProteinChip Reader (PBS-II, Ciphergen) according to an automatic data collection protocol with the following setting for the acquisition of data: high mass 200 kDa; digitizer rate 250 MHz; laser intensity 215, 245; sensitivity 10. The instrument was operated in positive ion mode with a source and detector voltage of 1.8 kV. Each spectrum was an average of at least 60 laser shots and exter-

nally calibrated with a mixture of seven known proteins ([Arg-8]-vasopressin, 1084.247 Da; somatostatin, 1637.903 Da; dynorphinA [209–225], 2147.500 Da; ACTH [1–24], human, 2933.500 Da; insulin B-chain, bovine, 3495.941 Da; insulin, human recombinant, 5807.653 Da; and hirudin BKHV, 7033.614 Da). Data interpretation was analyzed by the use of the ProteinChip Software (version 3.0).

2.5 RP-HPLC purification

A Waters 2695 HPLC (Waters Associates, Milford, MA, USA) was used with an auto-sampler and photodiode array detector. The analytical column was a Delta PAK C18 (5 µm particle size, 300 Å pore size, 150 mm × 3.9 mm, Waters Associates). Gradient elution started at a combination of 80% A and 20% B (elution buffer A: 0.02% TFA and 0.1% acetic acid in water; elution buffer B: 0.02% TFA and 0.1% acetic acid in ACN). The proportion of elution buffer B was increased linearly from 20 to 30% in 10 min, from 30 to 50% in 60 min, from 50 to 90% in 10 min, and from 90 to 98% in 20 min. The flow rate was 0.2 mL/min.

2.6 Tryptic digestion

The digestion protocol was used according to the methods in a previously published study [25]. Briefly, the collected LC fraction was concentrated to 10 µL, reduced with 200 mM DTT, alkylated with iodoacetamide, and digested with trypsin. The resulting solution was concentrated to 10 µL by freeze-drying for further characterization.

2.7 Protein identification by nano-LC-nano-ESI-MS/MS

The peptides were analyzed using TOF-MS and MS/MS with a nano-LC (DIONEX, LC Packings, Sunnyvale, CA) coupled with nano-ESI-MS/MS (Applied Biosystems, Q-Star XL, MDS Sciex, Concord, Ontario, Canada). The 10 cm × 75 µM id microcapillary LC column was self-packed using PicoFrit (New Objectives, Woburn, MA). This column had an integrated spray tip which was directly coupled with the nano-spray interface (Protana, Odense, Denmark) into ABI's Q-TOF mass spectrometer. The packing material was Luna C18, 3 µM, 100 Å (Phenomenex Torrance, CA). Samples were loaded onto a trapping cartridge (C18, 0.3 mm × 5 mm, from DIONEX, LC Packings) from Famos autosampler (DIONEX, LC Packings) at 30 µL/min. After a 5 min wash with ACN/water (2:98 v/v with 0.1% formic acid), the system was switched (Switchos, DIONEX, LC Packings) into line with the C18 analytical capillary column. Using an Ultimate solvent delivery system (DIONEX, LC Packings), a linear gradient of ACN (0.1% formic acid) from 20 to 95% over 34 min at flow rate of ∼300 nL/min was used to analyze the tryptic digests. Data was acquired using information-dependent acquisition (IDA) mode with Analyst QS software (Applied Biosystems).

The rabbit (taxonomy: *Oryctolagus cuniculus*) protein database was pre-extracted from the NCBI nonredundant (National Center for Biotechnology Information) protein database and used for protein ID searching using MASCOT software (Matrix Science, UK). The mass tolerances were ± 0.30 Da for parent ions (MS) and ± 0.15 Da for fragment ions (MS/MS). Variable modifications of carbamidomethylation of cysteine and oxidation of methionine, together with a maximum of one missed tryptic cleavage site were used.

2.8 Peptide quantitation with iTRAQ

Aliquots (5 µL) of rabbit tears taken prior to day 0 and after corneal wounding at days 1–3 were reduced, cysteines blocked (using methyl methanethiosulfonate (MMTS)) and digested with trypsin as described in the iTRAQ protocol (Applied Biosystems). Tear samples taken prior to corneal wounding were labeled with iTRAQ reagent 114 and tear samples from post-wounding days 1–3 were labeled with iTRAQ reagents 115, 116, and 117, respectively. These four samples were then combined and analyzed by 2-D nano-LC-nano-ESI-MS/MS. 2-D nano-LC coupled with nano-ESI-MS/MS was used for the analysis. The first dimension was a SCX column (300 µM id × 10 cm porosity 10 S SCX, DIONEX, LC Packings) with ten steps of salt plug (20 µL injection) elutions (10, 20, 30, 40, 50, 75, 100, 250, 500, and 1000 mM ammonium acetate) all at a flow rate of 30 µL/min and using a loading solvent of 0.1% formic acid/ACN (95:5 v/v). The second dimension of the RP separation was the same as that described above, but with an 85 min elution time. Parameters for the nanospray and other instrumentation were set as follows: ionspray voltage (IS) = 2200 V, curtain gas (CUR) = 20, declustering potential (DP) = 60 V, focusing potential (FP) = 265 V, collision gas setting (CAD) = 5 for nitrogen gas, DP2 = 15. All data were acquired using IDA mode with Analyst QS software (Applied Biosystems). For TOF-MS survey scan parameters: 1 s TOF-MS survery scan in the mass range of 300–1200 Da followed by two product ion scans of 3 s each in the mass range of 100–1500 Da. The "enhance all" function was used in the IDA experiments. Switching criteria were set to ions greater than $m/z = 350$ and smaller than $m/z = 1200$ with charge state of 2–4 and an abundance threshold of >20 counts/s. Former target ions were excluded for 60 s. IDA collision energy (CE) parameter script was used for automatically controlling the CE.

2.9 iTRAQ data analysis

Data analysis for the iTRAQ experiments was performed using ProQUANT 1.1 software, together with ProGroup Viewer 1.0 (Applied Biosystems) and searched against NCBI protein database. The mass tolerance settings for peptide identification in ProQUANT searches were 0.15 Da for MS and 0.15 Da for MS/MS, respectively. The cut off for the confidence settings was at 75.

For the quantitative results, Pro Group reports ratios (*e.g.*, 115/114) and error factors (EF). The EF expresses the 95% uncertainty range for a reported ratio. The true protein ratio is expected to be found between the (reported ratio)/(EF) and the (reported ratio) × (EF) 95% of the time. To obtain an average of ratios for a particular protein from three sets of iTRAQ data, we used a weighted average calculation using 1/log EF as the weight. First, we converted the ratios to log space [log (ratio)], converted the EFs to log EFs and used the inverse of the EF as the weight. The weighted average in log space was calculated using the following formula:

Weighted average (log space) = Sum [log(ratio) × weight]/Sum (weight), where weight = 1/log EF.

The weighted average of the ratios were obtained after converting them out of the log space. Weighted SDs were also calculated to indicate the variation of the observations (see Supporting Information for more detail).

3 Results and discussion

Rabbit tear protein profiles prior to (day 0) and after corneal wounding (days 1–5) were mapped using SELDI-TOF-MS. Figure 1 depicts the comparison of SELDI-TOF-MS profiles of rabbit tears over the low mass range (<20 000 Da) using NP-20 (hydrophilic surface) and H4 (hydrophobic surface). The tear protein profiles are very similar between these two different chip surfaces. The only noticeable difference in this low mass region (3000–20 000 Da) is that H4 chip has an
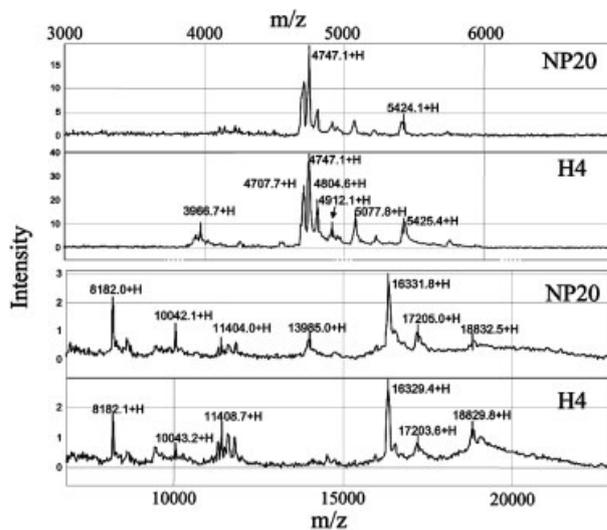

Figure 1. Typical SELDI-TOF mass spectra of normal rabbit tears on NP-20 hydrophilic surface and H4 hydrophobic surface. Many tear protein/peptide components were detected in the mass range of 3000–20 000 Da including some major tear proteins such as lipocalin and lipophilin around 16–19 kDa. Lipocalin and lipophilin have many isoforms. According to literatures [61, 62], 17.2 kDa peak and 18.8 kDa peak in the SELDI mass spectra are most likely rabbit lipophilin CL and one isoform of rabbit lipocalin.
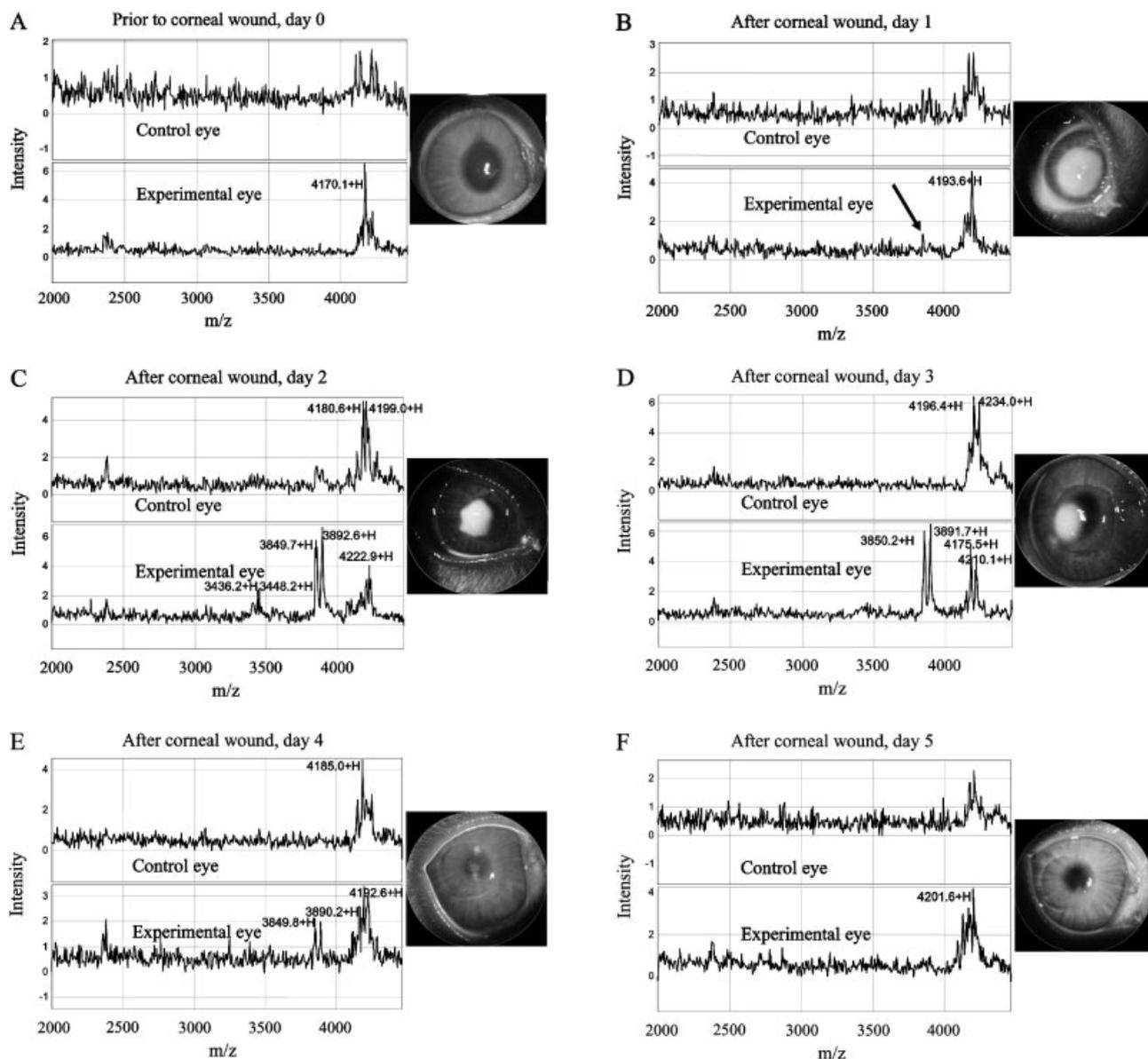
Figure 2. (A–F) A series of SELDI-TOF mass spectra (on NP-20 surface) in the range of 3000–4000 Da show the rabbit tear protein profile prior to day 0 and after corneal wounding (days 1–5). In each figure, the upper mass spectra represent fellow control eyes and lower mass spectra correspond to wounded eye. The corneal wound healing process was evaluated by staining the cornea with fluorescein and photographs were taken to document the wound healing process.

additional peak cluster around 4 kDa. However, tear protein profiles were quite different among ion exchange surface, hydrophobic, and Cu affinity binding surface [28, 30]. Figure 2A–F is a series of zoom-in SELDI-TOF-MS mass spectra (in the mass range of 3000–4000 Da) of rabbit tears from the fellow control eye and the experimental eye. Two peptides with the molecular sizes of 3850.0 ± 0.4 Da and 3892.2 ± 0.6 Da were significantly elevated in tears from the experimental eye on days 2–3 after wounding. The tear levels of these two peptides returned to baseline on postwounding day 5. Tears from contra-lateral, control eyes

showed no appreciable change in the amounts of these peptides over the 5-day period (Fig. 2A–F, upper trace). A preliminary database search based on only the molecular weight revealed that the mass weight of these two peptides are very close to those of two rabbit defensins, NP-1 and NP-2. Both hydrophobic (H4) and hydrophilic (NP-20) chips were used to profile rabbit tears. The results of a previous study from our laboratory showed that hydrophilic NP-20 chips were found to have strong binding to defensin molecules [25]. This finding is also supported by the work of other laboratories [36].

The application of an innocuous dye, fluorescein, which is used clinically to reveal cellular breaks in the structure of the corneal epithelium, revealed the progress of epithelial healing when viewed and photographed with a slit-lamp equipped with a cobalt blue filter. From the fluorescein stained photos, it was seen that the wound area (6 mm diameter) was revealed by fluorescein after the wound was created and that the staining area gradually decreased as the corneal epithelium gradually healed and regained the structural barrier property. Thus, the application of fluorescein demonstrated the reconstitution of the structural component of the innate immune system. This barrier is formed by junctional complexes around the outer surface cells of the cornea and is largely responsible for limiting corneal access of substances and pathogens from the tears. At day 5 after wounding, healing of the wound by the migrating epithelial cells was essentially complete as evidenced by a lack of staining (Fig. 2F).

In total 29 peaks were observed in the mass range of 3000–20 000 Da (on NP-20 surface). Changes in tear protein profiles following a corneal wound could be categorized into several different patterns (Fig. 3A–D). (A) For peaks at m/z 3436.2, 3448.2, 3850.0, 3892.2, and 11 368.4, the levels of these five proteins were elevated after corneal wound (day 1), reached peak values at day 2 or 3 and returned to baseline at day 4 or 5. Proteins with the molecular weight (m/z) 3850.0 and 3892.2 were the two most obvious changes (also see Fig. 2) and were chosen for detailed identification. (B) For peaks at m/z of 4172.3, 4707.5, 4804.9, 4911.7, 5072.8, 5219.0, 5424.1, 5736.9, 8182.1, 11 311.9, 11 615.5, 11 821.7, and 16 329.4, the levels of these 13 proteins were decreased after corneal wounding (days 1–3) and returned to baseline values by day 4 when wound healing was almost complete. Increased tear secretion and subsequent dilution of some tear proteins could underlie some of the loss in the levels of these peptides, but this would be more likely to occur early after wounding. This represented the most common pattern of changes in the tear protein profiles after corneal wounding. (C) For peaks at m/z 4201.7, 4692.3, 4747.1, 10 045.4, 11 206.8, 11 408.7, 11 573.2, and 14 647.2, the levels of these eight proteins fluctuated during wound healing. (D) For peaks at m/z 4222.2, 17 203.6, 18 829.8, the levels of these three proteins were fairly constant throughout the process. The levels of 15 out of 29 tear proteins returned to baseline when corneal wound healing was complete at day 5.

To identify peptides which were putatively considered to be rabbit defensins, an LC separation method based on a RP C18 column was developed. In an earlier study of rabbit tear proteins, an RP HPLC with a C4 analytical column was used to profile the tear proteins [24]. Figure 4 shows an HPLC-UV chromatogram of the rabbit tear protein profile 2 days after corneal wounding. Mass spectra from LC-MS chromatograms suggested a cluster of defensin-related peptides at a retention time at 19.2 min (mass spectra not shown).

In order to further characterize the peak as a defensin cluster, we collected the fraction under this peak and injected it into a nano-LC-ESI-MS/MS to obtain a high resolution TOF-MS mass spectra (Fig. 5). Four possible rabbit defensin peptides were suggested from the multiply charged ions, m/z 778.8922 ($[M + 5H]^{5+}$), m/z 770.3090 ($[M + 5H]^{5+}$), m/z 679.0946 ($[M + 6H]^{6+}$), and m/z 709.6131 ($[M + 5H]^{5+}$). After deconvolution, the experimentally determined masses compared well with those of rabbit defensins, NP-1, NP-2, NP-3B, and NP-5. The differences between experimental data and theoretical values are around 0.1–0.3 Da (Fig. 5).

For confirmation of the protein, the concentrated sample under the peak of Rt = 19.2 min was reduced, alkylated, and digested by trypsin. The resulting digests were further concentrated and analyzed by nano-LC-nano-ESI-MS/MS. A unique peptide fragment ion at m/z 365.20 (doubly charged ion) was selected by IDA for further fragmentation by MS/MS and the resulting spectrum is seen in Fig. 6A. The MS/MS signals matched y-ions, two b-ions, and one a-ion of a peptide fragment, ALC*LPR (C* represents cysteine modified with iodoacetamide to form carbamidomethyl-cysteine). A search of the NCBI protein database showed that the only match was with rabbit defensin NP-1. Another peptide fragment ion at m/z 552.76 (doubly charged ion) was also selected by IDA for further MS/MS fragmentation (Fig. 6B). The MS/MS signals matched seven out of seven y-ions, four b-ions of a peptide fragment, ALC*LPLER (C* represents cysteine modified with MMTS, the iTRAQ label added 144 Da to the N-terminal residue of the peptide). A MASCOT search against rabbit protein database showed that the rabbit defensin NP-2 was the only match.

The amino acid sequences of NP-1 and NP-2 are very similar (Table 1). The only difference between NP-1 and NP-2 is at residue position 13, where Arg is found for NP-1 and Leu for NP-2. Trypsin digestion of NP-1 and NP-2 generated a peptide fragment ALCLPR for NP-1 and ALCLPLER for NP-2. Other tryptic peptide fragments were also identified, *i.e.*, IHPLC*C*R (m/z 319.16, C* represents cysteine modified with iodoacetamide to form carbamidomethyl-cysteine) which are identical for NP-1 and NP-2. The sequence coverage for NP-1 and NP-2 was 39 and 45%, respectively (highlighted in Table 1).

The MS/MS spectrum of one unique peptide fragment (GFLC*GSGER, m/z = 558.19, +2, C* represents cysteine modified with MMTS, the iTRAQ label added 144 Da to the N-terminal residue of the peptide) that originated from NP-5 was also observed (Fig. 6C). However, it was not possible to obtain MS/MS signals for NP-3B and this was probably due to either low sensitivity or poor fragmentation.

Defensin levels in tear fluid prior to and after corneal wounding were also determined with iTRAQ experiments. iTRAQ technology is a relatively new protein quantification method which allows the analysis of four samples to be obtained simultaneously. The quantification was based on one unique peptide fragment (ALCLPLER), which originated from rabbit NP-2. The relative quantification that can be carried out with iTRAQ method is demonstrated in Fig. 6B by comparison of the peak area of 114 (prior to corneal wound-
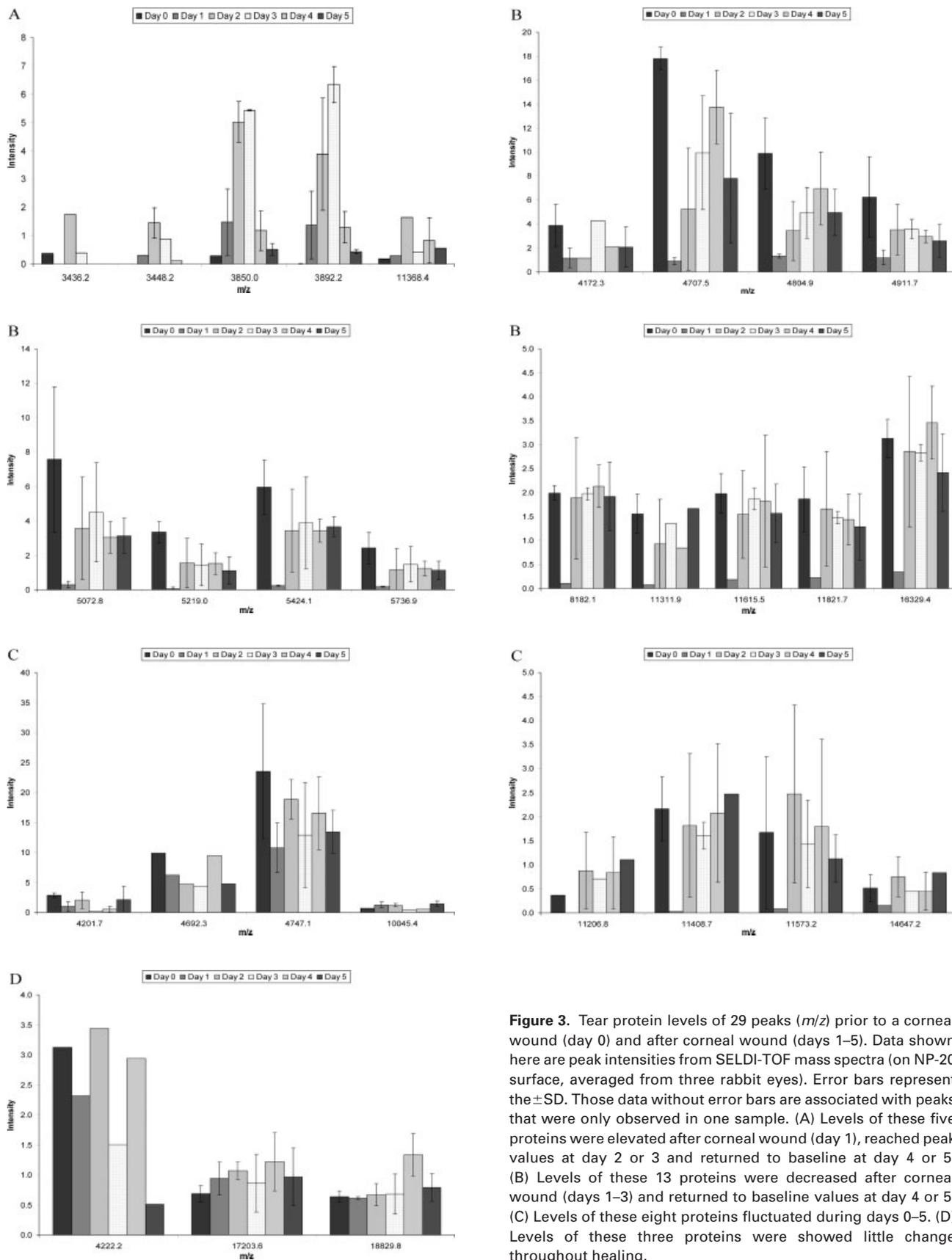
Figure 3. Tear protein levels of 29 peaks (*m/z*) prior to a corneal wound (day 0) and after corneal wound (days 1–5). Data shown here are peak intensities from SELDI-TOF mass spectra (on NP-20 surface, averaged from three rabbit eyes). Error bars represent the ±SD. Those data without error bars are associated with peaks that were only observed in one sample. (A) Levels of these five proteins were elevated after corneal wound (day 1), reached peak values at day 2 or 3 and returned to baseline at day 4 or 5. (B) Levels of these 13 proteins were decreased after corneal wound (days 1–3) and returned to baseline values at day 4 or 5. (C) Levels of these eight proteins fluctuated during days 0–5. (D) Levels of these three proteins were showed little change throughout healing.
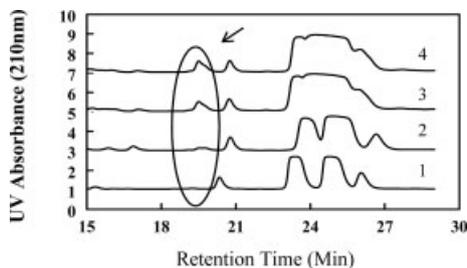
Figure 4. HPLC chromatograms of rabbit tears after corneal wounding (day 2) on a C18 column. The peak containing rabbit defensins (at 19.2 min) was collected and concentrated for further identification using high resolution MS/MS. Trace 1: prior to corneal wounding, day 0; trace 2, 3, 4: after corneal wounding, days 1–3.

ing, day 0), 115 (day 1 after wounding, day 1), 116 (day 2 after wounding, day 2), and 117 (day 3 after wounding, day 3). Major changes in the differential expression levels of NP-2 in tear fluid prior to and after corneal wounding were as follows (Table 2): 6.35 (115/114 ratio, day1 after wounding, weighted average from three rabbit samples, weighted SD: 1.12), 10.23

(116/114 ratio, day 2 after wounding, weighted average from three rabbit samples, weighted SD: 1.05), and 7.94 (117/114 ratio, day 3 after wounding, weighted average from three rabbit samples, weighted SD: 1.14). For comparison, the expression level of an abundant tear protein, lipophilin AL is also reported here (Table 2): 0.87 (115/114 ratio, day 1 after wounding, weighted average from three rabbit samples, weighted SD: 1.09), 0.93 (116/114 ratio, day 2 after wounding, weighted average from three rabbit samples, weighted SD: 1.08), and 1.02 (117/114 ratio, day 3 after wounding, weighted average from three rabbit samples, weighted SD: 1.03). These results support previous observations that showed lipophilin tear levels to be statistically unchanged after wounding when compared to the levels prior to wounding [24]. The NP-2 levels measured by iTRAQ (Fig. 6B, insert) correlated well with those obtained by SELDI (Fig. 2). We did not report NP-1 levels by iTRAQ here because we did not detect the unique peptide fragment signal of NP-1 (ALC*LPR) in some of the samples, whereas NP-2 signals were consistently captured in each sample. The reason could be that the unique peptide fragment of NP-1 (ALC*LPR) is more difficult to capture than that of NP-2 (ALC*LPLER).
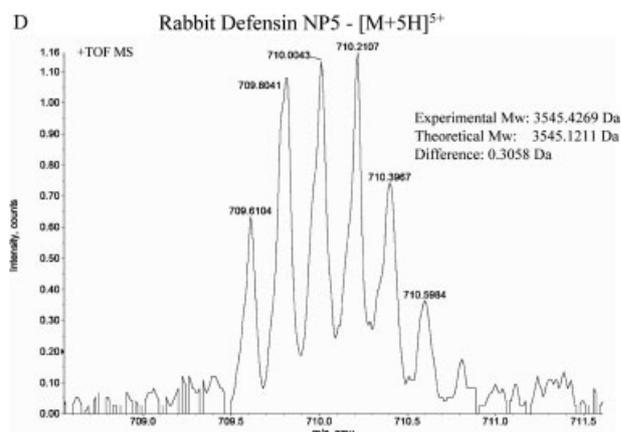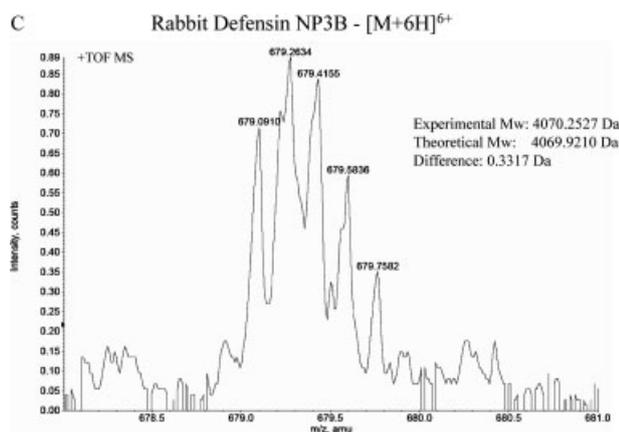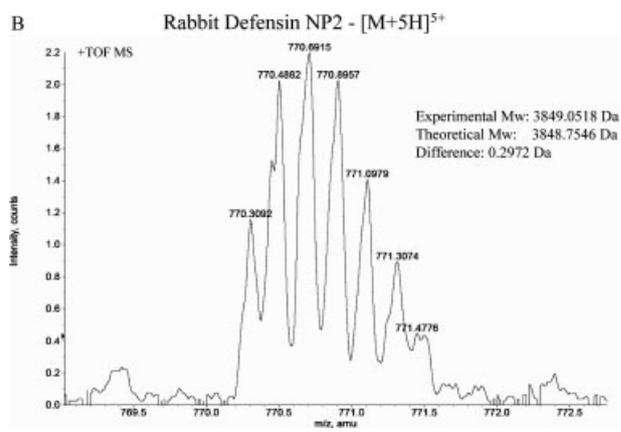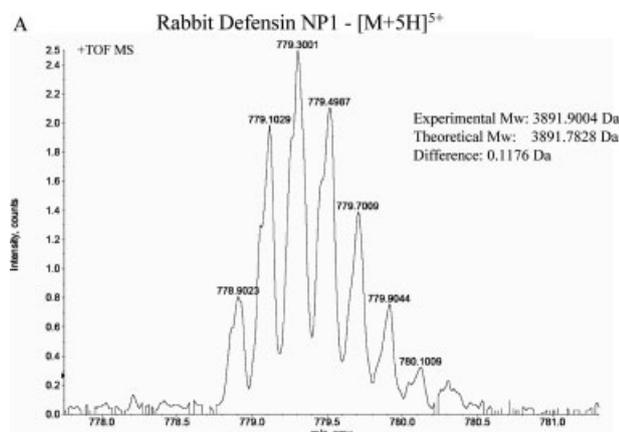

Figure 5. (A–D) High resolution TOF-MS spectra show the presence of rabbit denfensins, NP-1, NP-2, NP-3B, and NP-5 in the HPLC fraction (Rt = 19.2 min).

A

ALC*LPR (m/z=365.20), 2+

C*: Carbamidomethyl

365.2, [M+2H]²⁺

#	a	b	y
1			175.12
2	157.13	185.13	272.17
3	317.16	345.16	385.26
4	430.25	458.24	545.29
5	527.30	555.30	658.37
6			

B

ALC*LPLER, (m/z 552.76), 2+

C*: MMTS

#	b	y
1	216.15	175.12
2	329.23	304.16
3	478.23	417.25
4	591.31	514.30
5	688.36	627.38
6	801.45	776.38
7	930.49	889.46
8		

[M+2H]²⁺

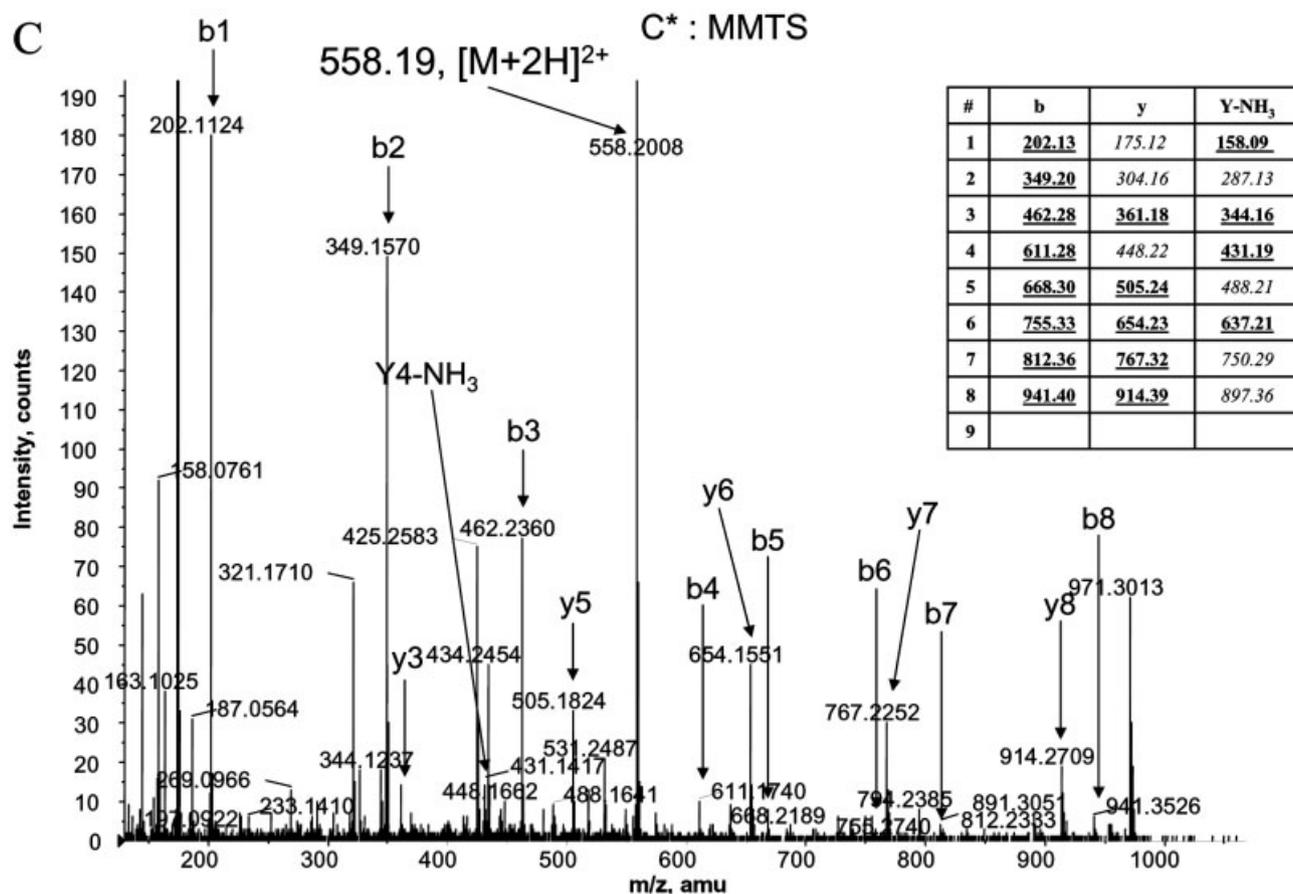| #	b	y	Y-NH$_3$
1	202.13	175.12	158.09
2	349.20	304.16	287.13
3	462.28	361.18	344.16
4	611.28	448.22	431.19
5	668.30	505.24	488.21
6	755.33	654.23	637.21
7	812.36	767.32	750.29
8	941.40	914.39	897.36
9			

Figure 6. (A) MS/MS spectra show the fragment ions from a doubly charged precursor ion *m/z* 365.2 originating from rabbit defensin NP-1. (B) A doubly charged precursor ion *m/z* 552.76 originating from rabbit defensin NP-2. Zoom-in view (insert) of low *m/z* region shows the relative abundances of rabbit NP-2 in tear fluid at day 0 (labeled with 114 reagent) and after corneal wounding day 1 (labeled with 115 reagent), day 2 (labeled with 116 reagent), and day 3 (labeled with 117 reagent) using iTRAQ quantification. The quantification is based on one unique peptide fragment (ALCLPLER) originating from rabbit NP-2. (C) MS/MS spectrum shows the fragment ions from a doubly charged precursor ion *m/z* 558.19 originating from rabbit defensin NP-5.

Table 1. Amino acid sequences of rabbit defensins NP-1 and NP-2

Name	Amino acid sequence	MW (Da)	Disulfide bridge positions
NP-1	VVCACRR**ALCLPR**ERRAGFCRIRGRI**HPLCCR**R	3891.8	3–31, 5–20,10–30
NP-2	VVCACRR**ALCLPLER**RAGFCRIRGRI**HPLCCR**R	3848.8	3–31, 5–20,10–30

Defensins are small cationic antimicrobial peptides with 29–45 amino acid residues and molecular weight around 3–6 kDa. Usually they possess six cysteine residues forming three intramolecular disulfide bonds, with at least two excess positive charges due to lysine, arginine, and histidine residues with a composition of about 30–50% hydrophobic amino acids. Six rabbit neutrophil α-defensins are known [37], NP-1, NP-2, NP-3A, NP-3B, NP-4, and NP-5. α-Defensins are generally found in high abundance in granulocytes and in other immune cells such as polymorphonuclear (PMN) cells at mucosal surfaces. Six human α-defensins

HNP-1, HNP-2, HNP-3, HNP-4, HD-5, and HD-6 have been identified so far with HNP1–4 from PMNs and HD 5–6 originating from intestinal Paneth cells [38].

Defensins show broad antimicrobial activity against bacteria [39–43], fungi [44, 45], and certain viruses [46, 47]. Other than their antimicrobial activities, many novel biological properties have been suggested by recent studies including stimulation of cell proliferation [48], as a chemoattractant for immune cells [49], stimulation of cytokine expression [50, 51], and antitumor activity [52]. Understanding corneal wound healing is important so that patients obtain optimal

Table 2. Using iTRAQ, the relative expression of NP-2 and lipophilin AL in rabbit tears was obtained prior to and after a corneal wound

iTRAQ ratio	Day 1/day 0 (115:114)	Day 2/day 0 (116:114)	Day 3/day 0 (117:114)
NP-2 (weighted average ratio)	6.35	10.23	7.94
NP-2 (weighted SD)	1.12	1.05	1.14
Lipophilin AL (weighted average ratio)	0.87	0.93	1.02
Lipophilin AL (weighted SD)	1.09	1.08	1.03

Weighted average ratios and weighted SDs (Sw) are given. The true average protein ratio is expected to be found between the (average ratio)/(Sw) and the (average ratio)*(Sw) 95% of the time. Day 0, before wounding; day 1, 1 day after wounding; day 2, 2 days after wounding; day 3, 3 days after wounding.

visual clarity after trauma or surgical procedures. Although more work is required to validate the concept defensins levels may be a useful peptide to monitor to determine if the corneal barrier function is intact.

The experimental corneal wound used in this study has similarity to the refractive surgical procedure, photorefractive keratectomy, in that the epithelium is removed and must heal over the denuded corneal surface [53]. Moreover, wound healing in general has common pathways which are activated upon injury whether it is by accidental trauma or from a planned surgery [2].

The normal cornea is an avascular and transparent tissue. Following injury, the neurogenic component of the response is rapidly deployed resulting in the release of PMNs onto the ocular surface where they would have the opportunity to release their granules containing defensins into the tears [13]. PMN infiltration onto the ocular surface usually occurs within a few hours following a corneal wound [54, 55]. However, considerable numbers of PMNs still can be found in the stroma even after re-epithelialization is complete [54, 56]. In a very recent study of neutrophil emigration in response to corneal epithelial abrasion in a mouse model by Li *et al.* [56], they reported that re-epithelialization was typically complete at 24 h but the neutrophil emigration returned to baseline at 48 h. However, the present study found that defensin levels in the tears dropped to baseline level after corneal re-epithelialization is complete. It is suggested that some of the tear defensins may have been from PMNs in the stroma and after the epithelium covered the stroma the proteins no longer had free access to the tears. The barrier function of the epithelial cells exclude proteins from entry into the cornea/stroma from the tears and very likely restrict the movement of peptides from the stroma into the tears.

We observed a similar temporal correlation of the levels of tear defensins and wound healing in our previous study [25] on human α-defensin levels (HNP-1, HNP-2, and HNP-

3) after ocular surface surgery; however, due to the constraints associated with human studies it was not possible to make a tight temporal correlation between healing and defensin levels in the tears. In that study, the concentrations of HNP-1, HNP-2, and HNP-3 reached more than ~10 μM at days 2–3 after surgery. By comparing the SELDI signal intensity of rabbit NP-1 and NP-2 after an experimental corneal wound with those of human HNP-1, HNP-2, and HNP-3 after an ocular surgery, the amount of rabbit defensins NP-1 and NP-2 could be estimated to be in a similar concentration range (~10 μM).

In this study, the structural component of innate immunity was compromised by removing the epithelial barrier function. However, with the breakdown of the physical barrier of the cornea, the eye becomes vulnerable to entry and infections from pathogens. Redundant naturally occurring biochemical systems attempt to provide protection in these situations with the presence of lactoferrin, and lysozyme (one of the most abundant proteins in human tears, but very low expression in rabbit tear fluids), and with augmentation by defensins. In contrast to NP-2, the expression of rabbit lysozyme in tears increases only transiently after an experimental corneal wound [24]. Therefore, after a corneal wound, the introduction of defensins (*i.e.*, NP-1 and NP-2 in rabbit) on the ocular surface will provide a more robust level of responsiveness of the local innate immune response. Synergistic effects with other antibiotic-like peptides and other tear anti-bacterial proteins such as lysozyme has been suggested [57]. Moreover, in addition to their antimicrobial activity, defensins may also promote wound healing by stimulation of cell proliferation and participate in mediating cytokine expression and chemoattraction of immune cells [44]. The exact mechanisms of action in these situations is not yet clear. However, a recent study from our lab showed that human defensins HNP-1 and HBD-2 have an important role in the biosynthetic and tissue remodeling responses of conjunctival fibroblasts [58]. It may be beneficial to introduce synthetic defensins [59, 60] during wound healing because as there is a high risk of infections when the epithelium is still not yet completely healed and in fact defensins may be useful to augment conventional antibiotics, which slow wound healing.

4   Concluding remarks

In this paper, we have shown that rabbit tear protein profiles changed dramatically after corneal wounding. Rabbit neutrophil defensins NP-1 and NP-2 were upregulated in the tears during re-epithelialization of an experimental corneal wound. The levels of NP-1 and NP-2 in the tears correlated with the course of wound closure reaching peak values at days 2–3 after wounding and returning to normal levels when the wound was re-epithelialized. The results of this study and our recent study in humans suggests that upregulation of defensins occurs after a sterile injury of the cornea

or the conjunctiva. This broadens the concept of the function of defensins. Defensins could have an important role in protecting the cornea from microbial attack and may also modulate wound healing processes as well. Defensins may also be useful to monitor the function of the corneal barrier. This study also demonstrated that iTRAQ technology can be applied for quantitative proteomics of the tears in a time-course study.

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