EXTENDED REPORT

Proteomic analysis of human meibomian gland secretions

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Background/aim: Human tears contain hundreds of proteins that may exert a significant influence on tear film stability, ocular surface integrity, and visual function. The authors hypothesise that many of these proteins originate from the meibomian gland. This study's aim was to begin to develop the proteomic methodology to permit the testing of their hypothesis.

Methods: Meibomian gland secretions were collected from the lower eyelids of adult volunteers and placed in a chloroform-methanol mixture. Samples were partitioned in a biphasic system and non-lipid phase materials were reduced, alkylated, and trypsin digested to obtain peptides for protein identification. This peptide mixture was separated by μ-capillary reverse phase chromatography and the effluent examined by nano-electrospray MS and data dependent MS/MS. SEQUEST software was used to identify proteins from the MS/MS spectra.

Results: The methodological approach to date has permitted the identification of more than 90 proteins in human meibomian gland secretions. Proteins include the α2-macroglobulin receptor, IgA α chain, farnesoid X activated receptor, interferon regulatory factor 3, lacritin precursor, lactotransferrin, lipocalin 1, lysozyme C precursor, potential phospholipid transporting ATPase IK, seven transmembrane helix receptor (also termed somatostatin receptor type 4), testes development related NYD-SP21 (also termed high affinity IgE receptor β subunit), and TrkC tyrosine kinase.

Conclusions: These findings indicate that the meibomian gland secretes a number of proteins into the tear film. It is quite possible that these proteins contribute to the dynamics of the tear film in both health and disease.

Human tears contain a complex array of proteins that exert a tremendous influence on tear film stability and ocular surface integrity.[1 2] To date, at least 500 proteins have been identified in the tear film.[3] These proteins, in turn, possess a vast range of functions, including bactericidal, virucidal, and fungicidal activities, specific and non-specific immune capabilities, and lipid transport abilities.[1] Human tear proteins may also minimise autolytic damage, attenuate ultraviolet B radiation induced effects, inhibit serine and cysteine proteases, promote ocular surface wound healing, and modulate the proliferation, motility, and differentiation of corneal and/or conjunctival epithelial cells.[1] The source of these proteins is traditionally thought to be the main and accessory lacrimal glands, the ocular surface epithelium, and the conjunctival blood vessels.[4]

However, we hypothesise that many of these proteins also originate from the meibomian gland. The rationale for our hypothesis is twofold. Firstly, the meibomian gland is a large sebaceous gland, and sebaceous glands are known to secrete a variety of proteins, such as IgA and pro-inflammatory cytokines (for example, TNF-α and IL-1α) in non-ocular sites.[5–10] Secondly, the meibomian gland secretes through a holocrine mechanism. Secretion occurs when mature epithelial cells lining the meibomian ducts disintegrate and release their proteinaceous lipid contents into the ducts and onto the ocular surface.[4] These meibomian gland proteins may represent a significant source of tear film proteins.

The aim of this study was to begin to develop the proteomic methodology to permit the testing of our hypothesis.

MATERIALS AND METHODS

Human subjects

Meibomian gland secretions were obtained from adult male and female volunteers, as previously described.[11] Briefly, secretions were collected from each eye by applying gentle pressure against the lower eyelids and collecting the excreted fluid with a curette. Individual samples were placed in glass tubes containing a 2:1 mixture of chloroform-methanol, and these tubes were then capped and stored at −70°C until experimental use. These studies were approved by the human studies committee of the Schepens Eye Research Institute (Boston, MA, USA) and were performed in accordance with guidelines established by the Declaration of Helsinki.

Biochemical and analytical methods

To evaluate meibomian gland secretions for proteins, seven different samples (labelled Q, R, U, V, W, X, and Y) were sonicated, exposed to aqueous ammonium acetate and partitioned in a chloroform-methanol-water biphasic system. The upper, non-lipid phase materials were collected, evaporated in a vacuum centrifuge and redissolved in 8 M urea containing 0.4 M ammonium bicarbonate. The mixture was reduced with dithiothreitol, alkylated with iodoacetamide, and digested in varying concentrations of porcine trypsin (Promega) to generate peptides for microcapillary (μ) HPLC/tandem mass spectrometry (MS)/MS protein identification. The protein digests were analysed by using a ThermoFinnigan LCQ ion trap mass spectrometer equipped with a Rheos 2000 micro-HPLC solvent delivery system (LEAP Technologies). Aliquots (that is, 25%–50%) of the digested samples were separated on 75 μm×10 cm Poros R2 (Applied Biosystems) packed in a PicoFrit column (New Objectives). The column was eluted with a 40 minute (samples Q, R, U, and V), 125 minute (samples X and Y), or 140 minute (sample W) gradient from 2% acetonitrile to

Abbreviations: IL, interleukin; MS, mass spectrometry; TNF, tissue necrosis factor

90% acetonitrile containing 0.1% formic acid. The gradient was generated at 50 μl/min and split immediately before the column to approximately 300 nl/min. Positive ion nanospray (spray voltage 2 kV), data dependent acquisition was used with one full scan from mass/charge (m/z) ratios 410–1200. This step was followed by product ion scans of the three most intense ions with dynamic exclusion of 3 minutes and collision induced dissociation energy at 33%.

The SEQUEST[12–14] software program (Bioworks 3.1, ThermoElectron) was used to identify proteins from the MS/MS spectra by searching a human subset of a non-redundant protein database from the National Center for Biotechnology Information (NCBI). The SEQUEST parameters (X_{corr}, ΔCn, Sp, RSp, %Ions) were adjusted to yield less than 1% false positives, by following reported procedures.[15] The false positive identification rate was estimated by creating a new database composed of the original database plus the reversed (nonsense) proteins, which was then searched with variations in the parameters until the number of false positives was less than 1% using the following formula:

$$\% \text{ false} = 2[n_{rev}/(n_{rev} + n_{real})]$$

A different set of SEQUEST parameters was developed for each sample. Resultant proteins were manually filtered and accepted only if they had an Sp value >350 or had a good quality MS/MS spectrum (that is, fragment ions definitely above baseline noise). These proteins were then categorised as the stringent criteria group.

The human subset protein database was then re-analysed using less stringent criteria, with a cross correlation (X_{corr}) >1.3 and delta correlation (ΔCn) >0.82. The search results were manually filtered and accepted only if they exhibited good MS/MS spectra. This second set of proteins was categorised as the broad criteria group.

In both the stringent and broad criteria groups, the proteins identified by one peptide were manually evaluated according to a recommended protocol.[16] The peptide had to satisfy several criteria. Firstly, the X_{corr} value had to be >1.5 for a +1 tryptic peptide or >2 for a +2 or +3 tryptic peptide. Secondly, the spectra of the fragment ions had to be above baseline noise. Thirdly, there had to be continuity to the b or y ion series (note: peptide fragments with charges on their N-terminal end are the ''b'' ions, and the ''y'' ions possess charges on the C-terminal end). Fourthly, the ion corresponding to fragmentation on the N-terminal side of proline had to be an intense ion.

In addition to these procedures, another preliminary method was used to examine meibomian gland secretions and involved one dimensional gel electrophoresis followed by μHPLC/MS/MS. Samples from different volunteers were placed in 0.9% saline, stored at –70℃, then thawed, reduced with 2-β-mercaptoethanol, denatured at 70℃, run on a precast TRIS-HCl 8–16% gel (BioRad), and stained with Sypro Ruby. This procedure demonstrated a series of molecular weight bands that were similar in each sample.

For analytical purposes, a single molecular weight band was excised from each of four lanes (that is, from four different samples) and these fragments were further processed at the Harvard School of Public Health Proteomics Facility (Boston, MA, USA). The gel samples were destained with NH_4HCO_3 in acetonitrile, alkylated with iodoacetamide, and digested with trypsin. Peptides were extracted from the gel fragments with formic acid and isopropanol, concentrated in a SpeedVac and resuspended in an aqueous solvent containing formic acid and acetonitrile. The digests were injected onto a C18 solid phase extraction trapping column (300 μm internal diameter×5 mm, Dionex, CA, USA) connected online to a self packed 75 μm internal diameter ×15 cm nano-LC reversed phase (Magic C18, 3 μm) PicoFrit column (NewObjective, MA, USA). The eluent was introduced into an LCQ Deca XP Plus mass spectrometer (ThermoElectron) by nanoelectrospray from a pulled fused silica 15 μm internal diameter tip of the nano-LC column. A 90 minute long gradient was applied on the column from a Surveyor HPLC system (ThermoElectron) to separate the digests. A solution of 0.01% formic acid and 2% acetonitrile in deionised water was used as HPLC solvent ''A,'' and an 0.01% formic acid, 5% isopropanol, and 80% acetonitrile mixture was used as HPLC solvent ''B.''

In data dependent MS/MS scanning mode, a full MS scan between 400 m/z and 1800 m/z was followed by five full MS/MS scans for the five most intense ions from the MS scan. Protein identification was performed with SEQUEST, searching the NCBI human database. The search was first set for at least a two peptide match and a ΔCn >0.08 and a Xcorr more

Table 1 Analysis of proteins in human meibomian gland secretions with stringent SEQUEST criteria

Accession No	Protein	Number of peptides	Samples
NP_067048.1	basic proline rich protein	1	Q
AAC82528.1	IgA α1 chain C region	3	Q
AAB59396.1	IgA α2 chain C region	2	Q
AAO11861.1	immunoglobulin kappa light chain variable region	1	Q
NP_001562.1	interferon regulatory factor 3	5	V, U
NP_006112.2	keratin 1	33	Q, U, V, W
NP_000414.2	keratin 2	15	W
NP_476429.1	keratin 3	1	W
AAH24292.1	keratin 5	13	W
NP_005545.1	keratin 6	11	U, W
CAA82315.1	keratin 9	13	U, W
NP_000412.2	keratin 10	2	Q, R, U, W, X
NP_005548.2	keratin 16	1	R
NP_009175.1	lacrimal proline rich protein	1	Q, R, U
NP_150593.1	lacritin precursor	1	R
AAH22347.1	lactotransferrin (lactoferrin)	3	Q
AAH15822.1	lactotransferrin precursor	3	Q
NP_002288.1	lipocalin 1	4	R
AAC63078.1	lysozyme C precursor	1	Q
AAH66594.1	similar to IgA C region	2	Q
XP_208403.1	similar to lipocalin 1 precursor	3	Q, R
BAC87456.1	unnamed protein product	3	Q

Human meibomian gland secretion samples (that is, Q, R, U, V, W, X, and Y) were processed with μHPLC/MS/MS procedures and the resulting ion spectra were filtered with stringent SEQUEST criteria. These parameters, as previously reported,[15] yield less than 1% false positives. The proteins are listed with their accession number, the number of peptides that matched, and the samples in which they were identified.

Table 2 Identification of proteins in human meibomian gland secretions with less stringent SEQUEST criteria

Accession No	Protein	Number of peptides	Samples
A41948	αfetoprotein enhancer binding protein	2	U, W
NP_001105.1	adenylate cyclase 7	1	W
NP_002323.1	α2-macroglobulin receptor	2	X, Y
NP_114121.1	CLLL8 protein	1	W
AAH15130.1	cytochrome c	2	U, W
CAA32479.1	dystrophin (AA 1-3685)[MASS = 10954]	2	R, V
AAN86577.1	EVC2 protein (Ellis-van Creveid syndrome)	2	W, X
Q96RI1	Farnesoid X activated receptor	2	Q, W
BAC03456.1	FLJ00397 protein[MASS = 16499]	1	W
AAA58477.1	fork head related protein[MASS = 36713]	1	Q
XP_297722.1	hypothetical protein	1	W
NP_710154.1	hypothetical protein FLJ31051	2	Q, U
T00391	hypothetical protein KIAA0612	1	W
XP_120509.1	hypothetical protein XP_120509.1	2	U, W
XP_296373.1	hypothetical protein XP_296373.1[MASS = 10954]	1	Q
XP_296857.1	hypothetical protein XP_296857	1	U
XP_299999.1	hypothetical protein XP_299999	2	Q, R
P01876	immunoglobulin[MASS = 37654]	1	Q
XP_290781.1	keratin 14	2	W
NP_149442.1	keratin associated protein 4–13	3	W, X
BAA31632.2	KIAA0657 protein[MASS = 135917]	1	Q
BAA74870.1	KIAA0847 protein[MASS = 70370]	1	W
BAA92565.2	KIAA1327 protein	5	Q, R, W
BAB47507.1	KIAA1878 protein[MASS = 48921]	2	W
Q16787	Laminin α3 chain precursor	2	Q
CAB51072.1	MSX2-interacting protein	2	W
AAK21295.1	NM-4	2	W
NP_005798	proteoglycan 4; megakaryote stimulating factor	1	R
BAC06129.1	seven transmembrane helix receptor	2	R, W
XP_225182.1	similar to AU RNA-binding protein/enoyl-coenzyme A hydratase	2	Q, W
XP_291583.1	similar to BarH-class homeodomain transcription	1	W
XP_215959.1	similar to chromosome 20 open reading frame 86	2	W
XP_236991.1	similar to colon cancer antigen NY-CO-45	2	W
XP_223599.1	similar to fidgetin-like 1	1	W
XP_224742.1	similar to GEM-interacting protein	6	W, X, Y
XP_208073.1	similar to H3 histone, family 3B	2	W
XP_222620.1	similar to hypothetical protein DKFZp434B1727	2	R, W
XP_228642.1	similar to hypothetical protein FLJ32191	2	Q, W
XP_230513.1	similar to hypothetical protein MGC14161	1	W
XP_215716.1	similar to hypothetical protein MGC27034	1	W
XP_066017.1	similar to keratin 18	1	U
XP_139603.1	similar to keratin 6 irs4	1	W
XP_237187.1	similar to KIAA0540 protein	1	W
XP_218841.1	similar to KIAA1233 protein	2	X
XP_027915.7	similar to KIAA1405 protein	3	U, W
XP_233885.1	similar to KIAA1805 protein	6	Q, U, W, Y
XP_219779.1	similar to KIAA2026 protein	2	R, W
XP_297211.1	similar to ladinin 1	1	R, W
XP_026998.2	similar to leucine rich repeat-containing 8	1	U
XP_071099.5	similar to olfactory receptor MOR262-2	1	Q
XP_290771.1	similar to RIKEN cDNA 3110023B02	2	Q, R
XP_232995.1	similar to ring finger protein 20	1	W
XP_236770.1	similar to topoisomerase (DNA) II binding protein	1	W
XP_231546.1	similar to zinc finger, imprinted 3	2	W
NP_477521.1	small breast epithelial mucin	1	R
NP_004644.1	SmcY protein (histocompatibility Y antigen)	3	W, Y
NP_710161.1	synaptonemal complex protein 3	2	R, W
NP_115986.2	testes developmental related NYD-SP21	1	W
NP_004771.1	transcription elongation factor A (SII)-like 1	1	U
I73633	TrkC tyrosine kinase	1	W
0501254A	Tro α1 H, myeloma	1	Q
NP_003106.2	UDP-N-acetylglucosamine pyrophosphorylase 1	1	W
BAB14689.1	unnamed protein product[MASS = 148993]	1	W
BAB70833.1	unnamed protein product[MASS = 32306]	2	R, W
BAC05042.1	unnamed protein product[MASS = 80996]	2	R, W
O75844	zinc metalloproteinase	1	W

Proteins in human meibomian gland secretion samples (that is, Q, R, U, V, W, X, and Y) were analysed using μHPLC/MS/MS techniques and broad SEQUEST criteria (that is, Xcorr >1.3, ΔCn >0.8). To decrease the possibility of false positives, all MS data were manually confirmed. If proteins were already identified with more stringent criteria (that is, table 1), they are not listed again in this table. The column headings are analogous to those in table 1.

than than 1.9, 2.2, and 3.5 for charge states of +1, +2, +3, respectively. The search was then set for at least one peptide match and a ΔCn >0.1 and a Xcorr more than 2.0, 2.5, and 3.75 for charge states of +1, +2, +3, respectively. The estimated rate of false positive identifications was below 0.6%.

RESULTS

Our methodological approaches, which have involved the use of different μHPLC/MS/MS procedures and analytical criteria, have permitted the identification of over 90 proteins in human meibomian gland secretions.

As shown in table 1, evaluation of various product ion spectra with stringent SEQUEST criteria, led to the identification of 22 proteins. These included IgA α chain, interferon regulatory factor 3, lacritin precursor, lactotransferrin, lipocalin 1, and lysozyme C precursor.

Relaxation of these criteria, in order to allow a broader assessment of protein composition, allowed the further identification of 66 proteins (table 2). These species included α2-macroglobulin receptor (also termed low density lipoprotein related protein 1), cytochrome c, farnesoid X activated receptor, laminin α-3 chain, seven transmembrane helix receptor (also termed somatostatin receptor type 4), testes development related NYD-SP21 (also termed high affinity IgE receptor β subunit), and TrkC tyrosine kinase.

During the course of these experimental studies, we also explored the possible use of 1D and 2D gels as a preparative step (for example, to remove lipids), before the utilisation of μHPLC/MS/MS methods. Our preliminary evaluation of selected bands from 1D gels resulted in the identification of five additional proteins (for example, potential phospholipid transporting ATPase IK) in human meibomian gland secretions (table 3). Furthermore, preliminary experiments with 2D gels permitted the characterisation of approximately 20 spots (data not shown). However, we discontinued the use of these preparative steps, given the reported difficulty in resolving hydrophobic (for example, membrane), very basic, and small proteins with these electrophoretic gels.[17]

DISCUSSION

This study demonstrates that human meibomian gland secretions contain over 90 proteins. This finding supports our hypothesis that many tear film proteins originate from the meibomian gland. Moreover, our results suggest that these proteins may exert a significant influence on tear film stability, ocular surface integrity, and visual function.

The identities of these proteins appear to reflect the holocrine nature of meibomian gland secretions, which should include cell membrane, mitochondrial, nuclear, cytoplasmic, and excreted materials.[4] For example, the secretions contained proteins that would typically be expressed in cell membranes, including: (a) the α2-macroglobulin receptor, which is a synonym for low density lipoprotein related protein 1, and may function in lipoprotein metabolism, as well as in proteinase and growth factor regulation; (b) TrkC tyrosine kinase, which is the receptor for neurotrophin-3; (c) potential phospholipid transporting ATPase IK, which may mediate phospholipid transfer; (d) testes development related NYD-SP21, which is another name for the high affinity IgE receptor β subunit.[18] This protein binds to the Fc region of the IgE receptor and is responsible for initiating the allergic response; and (e) seven transmembrane helix receptor, which is also called somatostatin receptor type 4.[18] This receptor inhibits adenylate cyclase, and also activates both arachidonate release and the mitogen activated protein kinase cascade.[18] It is possible that this receptor may mediate various actions of somatostatin in the meibomian gland, given that this tissue contains somatostatin positive innervation.[19]

The meibomian gland secretions also harboured: (a) a mitochondrial matrix protein, cytochrome c, which has a part in apoptosis; (b) a nuclear protein, farnesoid X activated receptor, which modulates cholesterol homeostasis; and (c) a variety of proteins that would invariably be secreted by other tissues, including laminin α-3 chain, lysozyme C precursor and lipocalin 1.[18 20] Laminin α-3 chain is a subunit of laminin-5 and is believed to be involved in cell adhesion, signal transduction, and keratinocyte differentiation.[18] Lysozyme C precursor possesses antibacterial activity.[1] Lipocalin 1 is a member of the lipocalin superfamily that binds a diverse array of lipophilic ligands (for example, fatty acids, phospholipids, retinol, and lipid peroxidation products) and is thought to act as a physiological scavenger of potentially harmful lipophilic molecules.[21]

A major question concerning our results is whether the meibomian gland secretions, which were collected very carefully, were in some way contaminated by adjacent lacrimal fluid. For instance, previous studies have identified the lacrimal gland as a major source of IgA α-chain, lysozyme, lactoferrin, lipocalin 1, and lacritin in the tear film.[1 22 23] However, several lines of evidence do not support this ''contamination'' possibility. Firstly, these proteins were not found in all meibomian gland samples, which would be anticipated if the secretions were admixed with lacrimal fluid. Secondly, we have discovered thousands of mRNAs in the mouse meibomian gland, including those, for example, related to MSX2 interacting protein, interferon regulatory factor 3, lactotransferrin (that is, lactoferrin), lysozyme C precursor, adenylate cyclase 7, dystrophin, transcription elongation factor A (SII) 1, and synaptonemal complex protein 3 (note: these mRNA data are available through the NCBI's Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) via series accession number GSE1582). We have also found low amounts of somatostatin receptor 4 mRNA in the mouse meibomian tissue. All of these proteins were identified

Table 3 Analysis of selected proteins in human meibomian gland secretions by use of 1D gels and modified μHPLC/MS/MS procedures

Accession No	Proteins identified	Number of peptides	Samples
XP_374101	hypothetical protein XP_374101	1	A4, E4
P04264	keratin 1	3 to 10	A4, C4, D4, E4
P35908	keratin 2	2 to 3	C4, E4
P13647	keratin 5	1	C4
P48668	keratin 6	1	E4
P08729	keratin 7	1	C4, E4
P35527	keratin 9	1 to 4	C4, E4
P13645	keratin 10	2 to 5	C4, E4
P13646	keratin 13	1	C4
P08779	keratin 16	1 to 2	C4, E4
	LOC144501 protein	1	C4
BC065721.1	lipocalin 1	2	A4
O60423	potential phospholipid transporting ATPase	1	C4

Four different samples (that is, labelled A, B, C, and D) of human meibomian gland secretions from young adults were electrophoresed in four lanes of a 1D gel, which led to the generation of multiple bands per lane. One similar molecular weight band was excised from each lane, processed with modified μHPLC/MS/MS procedures and analysed with SEQUEST criteria as outlined in the materials and methods). All band processing and analysis methods were repeated in order to confirm the protein data. Column headings are explained in table 1.

in our human meibomian gland secretions. Thirdly, lacritin synthesis is not unique to the lacrimal gland, but is now known to be produced by other sites, including conjunctival and corneal epithelial cells.[24] And fourthly, sebaceous glands have previously been shown to secrete IgA.[5]

It is intriguing to speculate how these meibomian gland proteins may impact the ocular surface and tear film. Lipocalin 1, for example, may have a role in the maintenance of tear film stability, and it is of particular interest that the tear levels of this protein, as well as lacritin precursor, are significantly reduced in meibomian gland dysfunction.[1 23 25 26] Lysozyme and lactotransferrin might serve anti-inflammatory and/or anti-microbial functions, whereas the laminin α-3 chain might influence corneal wound healing.[27 28] It is also possible that potential phospholipid transporting ATPase IK might act with other tear factors, such as catalytically active phospholipid transporting protein, to modulate lipid dynamics in the tear film.[29] That meibomian gland secretions might have these effects should not be surprising. Other ocular surface tissues, such as the lacrimal gland, also synthesise and secrete a number of proteins that influence tear film stability and corneal and conjunctival integrity.[26 30–33] Furthermore, some of these secreted proteins appear to promote both tear film destabilisation and ocular surface inflammation in dry eye patients.[31–34] How these lacrimal proteins may interact with those from the meibomian gland has yet to be determined.

The major goal of this study was to begin to develop the proteomic methodology necessary for the identification of proteins in human meibomian gland secretions. And we are just at the beginning of this methodological approach. Meibomian gland secretions present a number of experimental challenges, aside from the obvious need to remove the lipids. First and foremost is that fact that very low amounts (for example, 1 µl) of secretions may be obtained from a given individual, and the level of protein in these secretions is beneath the detection limit of a conventional protein assay (for example, Bradford, our unpublished data). Without protein concentration data, it is difficult to optimise procedures for removing salts, reducing possible interference by glycosylated peptides, or selecting ideal trypsin to protein ratios. In addition, we have attempted to use multidimensional protein identification technology to analyse the samples, but this approach has not yet yielded sufficient information to identify any tryptic peptides. As another consideration, we identified a number of keratins in meibomian gland secretions, but it is possible that these proteins originate from the skin. Keratins are known as a common contaminant in proteomic studies and are a particular problem with MS/MS procedures.[35]

Overall, our results indicate that µHPLC/MS/MS is an appropriate method for the analysis of proteins in human meibomian gland secretions. Ultimately, we believe that further methodological development may provide new insight into the dynamics of tear film proteins in both health and disease.

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These studies were approved by the human studies committee of the Schepens Eye Research Institute (Boston, MA, USA) and were performed in accordance with guidelines established by the Declaration of Helsinki.

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