Update on Human Tear Proteome

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Abstract

Tear film is a thin layer of non-newtonian fluid covering and protecting cornea and conjunctiva epithelia. The multifaced functions of tears are reflected by their complex structure and advances in proteomics/metabolomics/lipidomics technologies have greatly deepened the knowledge of the chemical composition of tears. The purpose of this article is to survey recent advances in proteomic analysis of human tears and to summarise the most relevant proteins proposed as biomarkers in dry eye. Besides, the potential clinical application of these biomarkers is discussed.

Keywords

Human tears, dry eye, proteomic analyisis

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Tears are a critical body extracellular fluid coating surface epithelial cells of cornea and conjunctiva and providing the optically smooth surface necessary for refraction of light onto the retina.

Production and quality of tears are controlled and co-ordinated by the lachrymal function unit (LFU), which consists of the main and accessory lachrymal glands, the ocular surface (including the cornea and conjunctiva), the meibomian glands and the interconnecting neural pathways.[1] A great body of past literature established that the conditions of LFU are reflected in the composition of tears.

Proteomics is the research area studying proteins expressed in a given biological compartment at a given time. The original definition of proteins as products of gene expression has lately included post-translational modifications of these gene products occurring in cell metabolism and turnover.[2] These arguments and the concept of temporal dynamics are of specific relevance in a body fluid as tears, directly in tiny equilibrium with the external environment and continuously exposed to both internal and external noxious agents.

Proteomic studies rely upon high-tech separation methods and equipment resolving the biological sample complexity, with the aid of procedures able to remove abundant proteins from samples (such as albumin and immunoglobulins in sera) and exhibit less abundant proteins. It is likely, however, that this pre-analytical step removes other possibly important protein biomarkers. An alternative option is to analyse body fluids closer to the site of interest: tears are a useful and accessible source for evaluating ocular surface tissue and lachrymal gland function in a number of disease conditions, such as dry eye, as well as treatment response.

Tears contain proteins, peptides, lipids, small molecule metabolites, electrolytes and an increasing attention in human tear proteome has been devoted in recent years, with the aim to develop biomarkers of disease.[3–5]

In this article we summarise the most relevant literature in proteomic analysis of human tears and point out those proteins proposed as biomarkers in dry eye. Potential application of tear proteomics in clinical setting and the related difficulties are also discussed.

Non-invasive Tear Sampling and Storage

Standardisation of tear collection was and still is a major hindrance for investigators. The main pitfalls are related to sampling of the needed amount of tears possibly avoiding unwanted dilution as a consequence of reflex production. It is recognised, in fact, that stimulated and unstimulated tears show different protein composition as demonstrated for secretory IgA and albumin.[6]

A comparison of different tear collection methods is provided in Zhou, 2012.[7] Methods currently available are based upon collection from Schirmer strips or various types of collectors (like sponges or rods positioned in the conjunctival meniscus to be impregnated by tears). Both are widely used methods but still unstandardised, since different cellulosic or polymeric materials are used, each having different adsorption, adhesive and releasing characteristics. In addition, many cell proteins can adhere (in particular, keratins) which may mask other protein peaks and quantification of proteins expressed /ml sample can turn to be difficult.

Perhaps collection by direct aspiration of either basal or flushed tears would be the most appropriate method. It can be accomplished through glass capillaries or a micropipette with a disposable sterile minitip at the outer conjunctival canthus.[8] The method is considered by many to be time-consuming, impractical and uncomfortable but after a short amount of training it can be safely performed by any operator. Tears contain various proteolytic enzymes, therefore appropriate storage is important to avoid sample modifications and unreliable results. It has been suggested that tears can be maintained without significant changes up to one week at 4 °C, up to two months at -20 °C, and up to four months at -70 °C before analysis.[9]

Analytical Methods of Tear Proteins

The first studies date back the early 1980s. In this pre-proteomic era researchers used mono-dimensional (1D) and bi-dimensional (2D) gel electrophoresis followed by immunoblotting detection, different high performance liquid chromatography (HPLC) techniques, reversed-phase (RP) chromatography, ion exchange chromatography, hydrophobic interaction chromatography (HIC) and gel permeation chromatography to separate tear proteins.

Major tear proteins were shown in human tears such as lactotransferrin, lysozyme, secretory IgA, zinc-α2- glycoprotein, exudated serum albumin, serotransferrin, IgG, lipocalins, cystatin SN, cystatin C, haptoglobin.

The first hi-tech equipments applied in tear proteomics were SELDI-TOF (surface-enhanced laser desorption ionisation - time of flight) and MALDI-TOF (matrix-assisted laser desorption ionisation - time of flight). A few microlitres of native tears can be directly applied (i.e. without any particular sample preparation) onto the SELDI array spots or a MALDI plates. Tear proteins are recognised with high sensitivity based on their physical or chemical characteristics, in particular the optimal mass range for both SELDI-TOF and MALDI-TOF is around 1,500 to 30 kDa and significant peak broadening is usually observed beyond 30 kDa.

Besides the typical tear major proteins already above listed, other proteins (particularly those small, low molecular weight ones) were recognised such as proline-rich protein 3 and 4[10,11] and lipophilin.[12,13] The coupling of SELDI/MALDI-TOF was also tried as a promising method to map tear protein profile, but the technique only provides the molecular mass and identification of species is then complicated and slow. In conclusion, the majority of the peptide peaks still remained unassigned.

HPLC as a method for peptide and protein separation was coupled with mass spectrometry (LC-MS/MS) through online electrospray ionisation (ESI); protein identification is then performed via database searching algorithms like Mascot[14] or ProtScore in ProteinPilot.[15] Using these and other sophisticated approaches of pre-fractionation, digestion and analysis up to around 500 tear proteins were recognised and reliably identified.[16]

Quantitative changes in protein expression is a fundamental further step to move to clinical applications. One of the latest proteomic approaches that allow simultaneously identification and quantification of proteins in one analysis is iTRAQ (isobaric tags for relative and absolute quantitation). This technique uses labelling of samples with isotope-coded tags and combination with LC-MS/MS yields to identification and quantitation of hundreds of proteins in different samples in one analysis.

A chapter apart in tear protein recognition is related to antibody protein array and multiplexed bead assays, high sensitivity technologies able to detect pg/ml levels of cytokines, chemokines, or growth factors that are still not routinely detected by the even most sensitive current mass spectrometry technologies. These low abundant metabolites are conventionally measured by ELISA (enzyme-linked immunosorbent assay) techniques, with the limitation to analyse only one or two species at the same time due to low tear sample size. A recently introduced cytometric beads assay (CBA) system[17] is able to quantify simultaneously up to 100 proteins (with a sensitivity of less than 10 pg/ml) by conjugating monoclonal antibodies on the surface of differently sized microspheres and the sample is then analysed by flow cytometry. Nearly 80 low abundant proteins in 50 µl of pooled tears were demonstrated by membrane-bound antibody array.[18] These high sensitivity techniques, however, are a targeted approaches dependent upon antibody specificity and availability, and they may be affected by interfering factors as conventional antibody-based ELISA.

Human Tear Proteome

Based on the past literature, it appears that normal human tears contain around 500 proteins, very recently extended to around 1,500 proteins, of which about 10 to 14 % are extracellular;[7,16] noteworthy to mention that extracellular proteins dominate in amount rather than number. Tear proteins can be classified according to their abundance, into: 1. major tear proteins at the high abundant region (concentration of mg/ml to µg/ml); 2. proteins secreted from ocular surface cells in the middle (µg/ml to ng/ml); 3. signalling molecules such as cytokines and growth factors at the low abundance region (ng/ml to pg/ml).

Major tear proteins comprise more than 90 % of the total amount and are secreted by the main and accessory lachrymal glands, Meibomian glands, conjunctival goblet cells or derive from leakage from plasma; these proteins include lysozyme, lactotransferrin, secretory immunoglobulin A (sIgA), lipocalin-1, lipophilin A and C, lacritin, proline-rich proteins, exudated serum albumin and serotransferrin. Proteins derived from ocular surface cells are normally secreted and released into tears during cell life and after cell damage and death. Tears can also contain proteins derived for foreign source such as an microbial organisms. The search for diagnostic biomarkers is focused in particular on proteins only released in the course of a particular disease process and considered peculiar for that condition.

In *Table 1* the most important proteins detected in tears are listed along with their proposed function in the LFU system.

Tear Proteins with Antimicrobial Activity

It is well recognised that lysozyme, lactoferrin and sIgA are fundamental components of the innate immune system of the eye in tears and represent a first-line protection against pathogens. Lysozyme and lactoferrin, in particular, are effective against a large spectrum of Gram-positive and Gram-negative bacteria by catalytic hydrolysis of the cell wall peptidoglycan the first[19] and by iron sequestration process from environment the latter. Lactoferrin is also active against fungi, yeasts, viruses and parasites.[20]

Secretory IgA (sIgA), rather than killing bacteria, inhibit their adherence onto ocular surface cells.[21] The level of sIgA expression appears regulated by the epithelial glycoprotein pIgR (polymeric immunoglobulin receptor) recognised in some proteomic studies, that transports dimeric IgA and strengthen sIgA immune functions.[22]

The biological functions of small proline-rich proteins in human tears are still undefined but it is hypothesised that they may be involved in the aggregation of microorganisms rather than direct killing as it occurs in oral mucosa defense mechanisms.[11]

Small, cationic peptides with anti-microbial activity such as defensins (α- and β-defensins)[23] and dermcidin[24] were identified in tears by proteomic studies. The amount of defensins in healthy tears is quite low, but it elevates dramatically (even 100 folds) following injury, to reach an effective anti-microbial activity based upon the electrostatic interaction between positively charged defensin molecules and the negatively charged microbial surface membrane.

Secreted GIIA phospholipase A2 (PLA2)[25] is another tear protein with recognised bactericidal activity, probably the most effective against

Table 1: Summary of the Major Tear Proteins

Protein	Swiss-Prot Entry name	Biological Process
Alpha-1-acid glycoprotein 1	A1AG1_HUMAN	Transport, acute phase response
Alpha-1-antitrypsin	A1AT_HUMAN	Blood coagulation, haemostasis
Alpha-enolase	ENOA_HUMAN	Glycolysis, transcription
Alpha and Beta-defensin family		Defense response
Aquaporin family		Transport
Calgizzarin	S10AB_HUMAN	Signal transduction, negative regulation of cell proliferation
Calgranulin-A	S10A8_HUMAN	Immune response, inflammatory response, apoptosis
Calgranulin-B	S10A9_HUMAN	Immune response, inflammatory response, apoptosis
Cystatin-C	CYTC_HUMAN	Defense response
Cystatin-SN	CYTN_HUMAN	Inhibitor
Dermcidin	DCD_HUMAN	Defense response
Haptoglobin	HPT_HUMAN	Defense response
IFN-gamma	IFNG_HUMAN	Immune response
IgG		Immune response
IgM		Immune response
Interleukin-1 alpha	IL1A_HUMAN	Inflammatory response, immune response
Interleukin-1 beta	IL1B_HUMAN	Inflammatory response
Interleukin-2	IL2_HUMAN	Immune response
Interleukin-4	IL4_HUMAN	Immune response
Interleukin-5	IL5_HUMAN	Immune response
Interleukin-6	IL6_HUMAN	Defense response, immune response
Interleukin-8	IL8_HUMAN	Immune response
Interleukin-10	IL10_HUMAN	Inflammatory response, immune response
Interleukin-17	IL17_HUMAN	Inflammatory response, immune response
Keratin-1	K2C1_HUMAN	Response to oxidative stress, complement activation
Keratin-2a	K22E_HUMAN	Keratinization
Keratin-9	K1C9_HUMAN	Intermediate filament organisation
Lacritin	LACRT_HUMAN	Positive regulation of secretion
Lactotrasferrin	TRFL_HUMAN	Defense response, immune response
Lipocalin 1	LCN1_HUMAN	Immune response
Lipophilin A	SG1D1_HUMAN	Regulation of steroid hormones
Lipophilin C	SG2A1_HUMAN	Regulation of steroid hormones
Lysozyme C	LYSC_HUMAN	Defense response
Matrix metalloproteinase family (MMPs)		Proteolysis
Mucin-1	MUC1_HUMAN	Membrane associated mucin
Mucin-2	MUC2_HUMAN	Secreted mucin, cell adhesion
Mucin-4	MUC4_HUMAN	Membrane associated mucin, cell adhesion
Mucin-5AC	MUC5A_HUMAN	Secreted mucin
Mucin-16	MUC16_HUMAN	Membrane associated mucin, cell adhesion
NGF	NGF_HUMAN	Inflammatory response
Phospholipase A2	PA21B_HUMAN	Lipid degradation, lipid metabolism
Polymeric Ig Receptor	PIGR_HUMAN	Immune response
Prolactin-inducible protein	PIP_HUMAN	Transport
Proline-rich protein 3	PRR3_HUMAN	Nucleic acid binding
Proline-rich protein 4	PROL4_HUMAN	Visual perception
S100 A4	S10A4_HUMAN	Epithelial to mesenchymal transition
Serotransferrin	TRFE_HUMAN	Iron transport
Serum albumin	ALBU_HUMAN	Transport
sIgA		Immune response
TNF-alpha	TNFA_HUMAN	Defense response, immune response
Trefoil factor 1	TFF1_HUMAN	Response to stress
Trefoil factor 3	TFF3_HUMAN	Defense response
Zinc-alpha-2-glycoprotein	ZA2G_HUMAN	Immune response

Gram-positive bacteria[26] by hydrolysis of the phospholipid component of the bacterial cell membrane. PLA2 is also able to kill Gram-negative bacteria with the concurrent action of a bactericidal/permeability-increasing protein[27] able to disrupt their lipopolysaccharide envelope.

Mucins

Mucins represent an important interface between the tear film and ocular surface and play a key role in the stability of tear film. Mucins are O-linked glycoproteins and are generally divided into: 1. gel-forming/secreted mucins; 2. soluble/secreted mucins; and 3. membrane associated mucins.[28] Both membrane associated mucins (MUC1, MUC4 and MUC16) and secreted mucins (MUC5AC, MUC2) were demonstrated in human tears.[29] The major gel-forming mucin of the ocular surface, MUC5AC, is secreted by the goblet cells of the conjunctiva and epithelial cells of the lachrymal glands and nasolachrymal ducts.

Glycoprotein 340 (gp340) is an abundant mucin-like protein secreted by lachrymal glands and detected at higher concentrations than other mucins (such as MUC4 and MUC5AC) in tears. It is suggested that gp340 protein plays antimicrobial activity by aggregating bacteria.[30]

Trefoil factor family (TFF) peptides non-covalently interact with mucins and affect their viscosity.[29] In humans, there are three TFF peptides and proteomic studies showed TFF1 and TFF3 in normal human tears.[29,31]

Lipid-loving Proteins

Tear lipocalin (TLC, previously called tear-specific prealbumin) is a major protein in tears as it comprises 15–33 % of the mass of protein in tears. TLC is a member of the lipocalin superfamily that is produced in the lacrimal gland as well as in von Ebner's gland. TLC binds to an extensive and different set of lipophilic molecules, it is suggested to play a role in the regulation of tear viscosity and may be a key factor in maintaining tear film stability by acting as a lipid scavenger.[32–34]

Tear lipophilins A and C belong to human uteroglobin superfamily; they are relatively abundant in tears and are believed to be steroid-regulated and steroid-binding molecules.[35,36] A decrease in tear TLC and lipophilins AC content in DE has been demonstrated and correlated to tear stability reduction.[33]

Tear Proteome in Dry Eye

Dry eye (DE) is one of the most frequent ocular surface disease observed in clinical practice. According to the International Dry Eye Workshop[37] DE is "a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear-film instability, with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface". It is widely recognised that a poor correlation or even conflicting results exist between clinical tests utilized to assess ocular surface status and subjective discomfort symptom complain. The search for more objective diagnostic tests is therefore an urgent need and the issue for a large body of investigations.

Early studies focused onto tear lysozyme or lactoferrin quantification as an indicator of lachrymal gland impairment as DE result. Decreased lysozyme or lactoferrin concentrations in tears of patients with dry eye of different severity (including Sjogren's syndrome patients) were demonstrated.[38] Decreased lysozyme and lactoferrin levels showed an higher diagnostic performance in SS-I diagnosis as compared to common clinical tests: positive predictive values (PPV) was found 13.8 for lactoferrin (cut-off ≤1.40 mg/ml) and 29.6 for lysozyme (cut-off ≤0.95 mg/ml), while it was 6.8 for Schirmer test I and 5.2 for break-up time (cut-off mm ≤5 /5 minutes and ≤10 sec, respectively).

Various proteomics approaches have been recently used to identify potential tear biomarkers able to unequivocally distinguish between DE and normal control populations. An increase of inflammatory related proteins (calgranulin A) and a decrease of protective proteins (lysozyme, proline-rich protein 3 and 4, nasopharyngeal carcinoma-associated proline-rich protein and α-1-antitrypsin)[10] were demonstrated in tears from DE patients; results showed sensitivity and specificity around 90 %.

Six up-regulated proteins (α-enolase, α-1-acid glycoprotein 1, calgranulin A and B, S100 A4 and calgizzarin) and 4 down-regulated proteins (prolactin-inducible protein, lipocalin-1, lactoferrin and lysozyme) were found to be associated with DE and identified as potential tear biomarkers for DE (diagnostic accuracy 96 % using a 4-protein biomarker panel).[15]

A significant decrease in amount of lactoferrin, lipocalin-1 and lipophilin A e C and a significant increase for serum albumin as an indicator of inflammation was shown in evaporative DE versus normal controls.[33] The enhanced susceptibility to ocular surface infections was suggested to be in direct relationship to reduced levels of lysozyme and lactoferrin in tears.[39]

Tear proteomic changes in course of Sjogren's syndrome (SS) were extensively analysed by many proteomic studies, showing that tear concentration of selective water channel protein aquaporin 5 was higher in SS dry eye than non-SS dry eye and controls,[40] the goblet cell mucin MUC5AC was significantly decreased in SS patients,[41] decreased levels of lactoferrin and lysozyme were also noted as in non-SS DE.[38,42–45] A diagnostic protein panel was suggested consisting of 10 different peaks (proteins not recognised)[46] to discriminate Sjogren syndrome, non-SS DE and controls.

Tear proteomic changes were also demonstrated in patients with blepharitis compared normal controls and down-regulation of serum albumin, α-1-antitrypsin, lacritin, lysozyme, prolactin inducible protein, cystatin was shown.[47]

In hyperthyroidism, ocular surface diseases such as dry eye are a common finding involving patients with a long history of the disease more frequently.[48] Proteomic analysis showed increase in lactoferrin and zinc-α2-glycoprotein[49] and in nerve growth factor (NGF)[50,51] in tears of patients vs controls.

Patients wearing contact lenses (CL) are likely to report DE symptoms, which is considered as the primary reason for CL intolerance and dropout. Patient's response to the presence of the lens can be investigated in tears, a substrate in which metabolic products from the underlying corneal epithelium are accumulated.

An alteration of tear protein profile in CL wearers has been demonstrated in the past literature with particular reference to sIgA and exudated albumin decrease,[52] with possible relationship to lens material and design.

The presence of pro-inflammatory and inflammatory markers in DE patients' tears is now an hallmark of DE disease. Tear cytokine profiles

was analysed by ultra-high sensitivity techniques and multiplexed bead assays and significant changes were demonstrated in DE patients versus control subject tears. In particular, increased levels of interleukin (IL) IL-1α and mature IL-1β [53], IL2, IL-4, IL-5, IL-6, IL-10, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, chemokine IL-8 [54-56], IL-17[57] were found in non-SS, SS meibomian gland disease (MGD) DE.

Interestingly, tear IL-6 levels were positively correlated with the severity of subjective discomfort symptoms.[58] IL-17, in conjunction with IL-1 and IFNγ, appears to stimulate the production of different matrix metalloproteinases (MMPs) by the corneal epithelium, with the subsequent failure of corneal barrier function.[57]

The Future – Proteomics From Bench to Bedside

Proteomics represents an outstanding selection of techniques able to compare protein profiles either qualitatively and quantitatively. Proteomic data can shed further insights into biological systems and identify promising markers candidate to become gold-standard diagnostic and innovative pharmaceutical research and development targets.

However, proteomics is still a difficult task under several points of view, due to the huge complexity of protein mixtures in a biological fluid. For biomarker research in many diseases, proteomics is still in the discovery phase to identify and quantify thousands of proteins in the samples. At this stage, analytical equipment are labour-intensive, sensitive to many processing-related variables, bioinformatics is required to integrate informations through time consuming approaches, equipment and consumables are still rather expensive. As a result, the route of proteomic research towards daily clinical practice application is still in progress in many fields including tear analysis.

While waiting for new discoveries from current proteomic studies, an emerging option in tear protein analysis appears the use of lab-on-chip microfluidic systems. Although limited in the number of protein solved in the same run (15–20 recognisable peaks), these systems have the unique characteristic to conjugate the use of a small sample (1–2 µl), practical and reproducible sample pre-analytical preparation, affordable time of analysis and reasonable costs. Many systems are now under progress[59,60] but to our knowledge at present only two are commercially available to separate and quantitate proteins from 5 to 250 kDa (Bioanalyzer 2100, Agilent Technology, Waldbronn, Germany and ExperionTM Automated electrophoresis station, Bio-Rad Laboratories Inc, Hercules, CA, US). The separation of the major tear proteins has been demonstrated[61,62] and validated[63] with the Bioanalyzer 2100, this system also offers the possibility to build up a protein panel for DE diagnosis that was shown to display an high diagnostic performance.[64]

A very recent point-of-care system (RPS InflammaDry Detector, Rapid Pathogen Screening, Inc., Sarasota, FL, USA) has been proposed to rapidly assess the inflammatory marker MMP-9 in a reliable way, able to diagnose DE with high accuracy.[65] ■

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