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Tear biochemistry: a review

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Introduction

The tear layer has been subdivided into three separate layers: the outer oily layer, the central aqueous/ watery phase and the inner mucous layer, each being produced by different glandular or cellular systems. Several functions have been attributed to the tear layer: to form a smooth optical surface on the cornea, to lubricate the cornea and conjunctiva, to provide anti-bacterial activity, to keep the cornea moist, to serve as a conduit for the entry of polymorphonuclear leucocytes into the cornea and to remove toxic substances from the ocular surface[1, 2]. The oily layer of the tears is produced primarily by the meibomian glands with the glands of Moll playing a role[1]. The meibomian glands (also known as the tarsal glands) are long sebaceous glands that are found in both the upper and lower lids. These glands are situated in the tarsal plates and number approximately 25 in the upper lid and 20 in the lower lid. The openings of the glands can be seen on the lid margin[3]. The glands of Moll can be considered as under- developed sweat glands that run parallel to the bulbs of the cilia[3]. The lacrimal gland and the accessory glands of Krause and Wolfring produce the middle aqueous layer of the tears. The lacrimal gland has two separate portions: a large orbital portion and a smaller palpebral portion. The lacrimal gland accounts for approximately 95% of the aqueous phase of the tears[2]. New evidence suggests, however, that the aqueous layer only makes up 60% of the thickness of the tear layer[4]. The orbital portion of the gland can be found in a fossa in the lateral roof of the orbit while the palpebral portion is placed just above the lateral section of the upper fornix[3]. The glands of Krause have the same structure as the lacrimal gland and are placed in the subconjunctival tissue of the upper fornix. The glands of Wolfring are larger than the glands of Krause and are situated in the upper lid along the upper border of the tarsus[3]. The mucous layer of the tears is produced by the goblet cells of the conjunctiva. Goblet cells can be found in all areas of the conjunctiva and are large oval or round cells[3].

The tears contain numerous components. Broadly speaking proteins (mucins, enzymes, glycoproteins, immunoglobulins etc), lipids, electrolytes, water and organic solutes are to be found in the tear layer[1-4]. The aqueous layer of the tear film contains predominantly proteins, water and electrolytes[5] while the outermost oily layer contains several classes of lipids (for example: wax esters, triglycerides, free fatty acids and polar lipids) and neutral diesters[6-7] and the inner-most mucin (glycoproteins) layer contains mucin types that are considered to be secreted or membrane-associated[8-10].

The lacrimal gland receives innervation primarily from the parasympathetic nervous system and is regulated mainly by the cholinergic fibres of this system, however, adrenergic sympathetic stimulation, peptide agents and humoral factors all play a role in modulating the processes of this gland[5-7]. The regulation of the lacrimal gland has proved to be an exceptionally complex process[2]. Afferent sensory nerves originating in the cornea and conjunctiva may be stimulated by mechanical, thermal and/or chemical stimuli which in turn result in stimulation of the lacrimal gland[5]. Light, via the optic nerve, can also stimulate the lacrimal gland[5]. Epidermal growth factor (EGF), a member of the growth factor family, has been shown to stimulate and modify the neural regulation of the lacrimal gland[5, 11]. The neurotransmitters released by the para-

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sympathetic and sympathetic nerves have been shown to be the most powerful stimuli of lacrimal gland function[5]. The detailed biochemical pathways involved are beyond the scope of this review. The interested reader is referred to a number of detailed reviews for an in-depth discussion of these pathways[5, 11-13].

Meibomian gland regulation seems to be dual natured[14] (at the least). Neural influences have been shown by several researchers[15, 16]. The cholinergic system has been suggested as being involved in the regulation of human meibomian glands[15]. This view is supported by others[16-18] who have shown results (investigating rat meibomian glands) strongly suggesting that the parasympathetic nervous system modulates the functioning of the meibomian glands. A further regulating influence on the meibomian gland is androgens[19-21]. An androgen is: "any number of hormones, such as testosterone, that influence the development of the male reproductive system"[22]. Androgens appear to play a role in stimulating the synthesis and secretion of lipids from the meibomian glands, however, the exact mechanism by which this process is regulated is not clear at present[20]. Sullivan *et al*[20] have shown that androgen deficiencies may result in altered lipid patterns in human meibomian gland secretions.

The regulation of conjunctival goblet cells is also a complicated process and only a superficial discussion will be presented in this review (the interested reader can consult excellent reviews on this specific subject by referring to Dartt[23, 24]). The conjunctiva has two layers, the stratified epithelial layer and the underlying stroma[3]. The epithelial layer has further been subdivided into five different cell types[25]. However, in this context epithelial cells are classified as goblet cells or stratified squamous cells. It has been shown that Ca2+ has an influence on cholinergic-induced goblet cell secretion[26]. Stimulation of the goblet cell can also be influenced by the enzyme protein kinase C (PKC) of which the conjunctival goblet cells contain seven different isoforms[26] ("catalytically and structurally similar but genetically distinct enzymes from the same organism"[24]). Goblet cells are surrounded by both parasympathetic and sympathetic nerve fibres[27] thus some autonomic influence might be inferred. Evidence has been provided to suggest that parasympathetic nerves, acting via acetylcholine and vasoactive intestinal polypeptide[28] (VIP), can stimulate goblet cell secretions[23]. Acetylcholine, however, does not stimulate stratified cells. PKC stimulates goblet cells but inhibits stratified cells[23]. Ca2+, on the other hand, stimulates both goblet cells and stratified cells[23]. The suggestion is that neural stimulation of conjuntival secretion depends on which nerves and which neurotransmitters are released[23, 24]. EGF also appears to play some role in goblet cell regulation[23].

The division of tear components into proteins, lipids and mucins is largely arbitrary yet also loosely related to the predominant components found in the three different layers of the tears. There is, admittedly, overlap. For example: all enzymes are proteins, the mucins are essentially glycoproteins, immunoglobulins are proteins and so on.

Proteins

Approximately 500 different proteins have been identified in the tears of the closed eye (de Souza *et al*[29] cited in Laurie *et al*[30]). Early electrophoretic investigations into proteins present in the tears revealed at least 60 different proteins[31]. Of the 60 proteins identified at least 20 were shown to be secreted by the lacrimal gland[31]. Furthermore the proteins present in tears were found to be products of a local synthesis process as opposed to originating from blood circulation[32].

The three principle proteins found in tears are lysozyme, lactoferrin and tear-specific prealbumin (lipocalin)[2, 32, 33]. Lysozyme is an enzyme (a protein) that is capable of destroying cell walls by breaking bonds (known as glycosidic bonds) between carbohydrate units in the peptidoglycans of the cell walls of bacteria[34, 35]. Tears, at least partially, have bacteriolytic activity due to the presence of lysozyme[36]. Lactoferrin too has antimicrobial activity as well as a possible role in ocular surface protection from free radicals[2]. The specific activity of tear-specific prealbumin (lipocalin) in tears is open to speculation at present[2]. However, it has been suggested that lipocalin has several important functions in the tear layer. Lipocalins may offer protection to the cornea by acting as clearance factors for harmful lipophilic substances[37] or they might contribute to the effective spreading of lipids over the tear film[38].

Using electrophoresis combined with immunologic identification, Coyle *et al*[39] showed the presence of

other proteins including albumin, IgA, IgG, secretory component and transferrin. Membrane-bound antibody arrays (MA) have been used to identify many other proteins in tears[40]. Epidermal growth factor (EGF), monocyte chemoattractant protein, tissue inhibitor metalloproteinase (TIMP), angiogenin, chemokines, cytokines, growth-related oncogene, epithelial neutrophil-activating protein and macrophage inflammatory protein have all been detected using MA[40, 41]. Growth factors, including EGF, are a family of factors that are able to regulate ocular surface wound healing, and corneal and conjunctival epithelial cell proliferation, differentiation, growth and development. EGF is also able to stimulate and modify lacrimal gland secretion. The EGF family are important regulators not only of the lacrimal gland but of the ocular surface as well[5]. Matrix metalloproteinases (MMP) are a family of zinc-dependant endopeptidases that are capable of degenerating all types of extracellular proteins[4]. They are also known to process bioactive molecules and to play a role in cell proliferation, migration, differentiation, angiogenesis and apoptosis[4]. The effects of MMP's can be counteracted by naturally occurring MMP inhibitors (TIMP)[4].

Immunoglobulins (antibodies) are glycoproteins that bind antigens (invading organisms) with a high specificity and affinity. There are five distinct classes of immunoglobulin (IgA, IgG, IgM, IgD and IgE). The immunoglobulins play an important part in the immune system[42]. Cytokines are protein or glycoprotein molecules that are secreted by cells in response to specific stimuli[42]. "Cytokines are multifunctional short-acting, short-range mediators of cellular activity that are released by T cells and other immune and non-immune cells"[4]. Cytokines play a critical role in inter-cellular communication and may induce growth, differentiation, chemotaxis and/or cytotoxicity[42]. Chemokines are a small group of cytokines with the express function of chemoattraction of lymphocytes, monocytes and neutrophils, all of which play a role in the immune response[42]. Angiotensin II (an octapeptide), a powerful vasopressor agent, formed by the action of angiotensin-converting enzyme, have both been isolated in human tears[43].The suggestion has been made that the presence of these agents implies some physiological function for angiotensin in the eye[43] (the exact function needs to be elucidated). Insulin and insulin-like growth factor have been isolated in the tears of human eyes[44]. Diabetes is known to be related to dry eye and altered ocular wound healing and the implication is that insulin may play a relevant role in ocular surface physiology[44]. Lacritin is another protein found in human tears. Its acts as a prosecretory mitogen (growth factor) that may play an important role in lacrimal secretion, and renewal of lacrimal and ocular surface epithelial structures[45].

Tear proteins are known to play a role in the antimicrobial and anti-inflammatory defences of the eye. They probably are also important in normal epithelial growth, protein/fluid/electrolyte secretion and many other aspects of normal ocular physiology, including (but probably not restricted to): angiogenesis, biosynthesis, calcium and carbohydrate metabolism, cell adhesion and motility, cell growth, anti-apoptosis, immunology and lipid/cholesterol metabolism[30].

Lipids

The exact roles that lipids play in the tear film are not completely agreed upon. However, the principal roles that lipids play are suggested as being the following[46, 47]: to act as a wettability barrier and contain the tears within the palbebral aperture, prevent maceration of the skin of the lids, to form a seal over exposed portions of the eye during sleep, prevent contamination of the tears by sebaceous lipids, reduce tear evaporation, impart stability to the tear layer, provide a smooth optical surface to the eye, provide some anti-microbial activity and to lower free energy of the tear film surface. The tear lipid layer is almost exclusively produced by the meibomian glands of the lids which are tubulo-acinar, holocrine in nature[47]. The major constituents of meibomian lipids include: wax esters, cholesterol and cholesterol esters (non-polar lipids) which make up 60-70% of the lipids found in the tears. Polar lipids are also present and are mainly phospholipids and glycolipids[46, 47]. Diesters, hydrocarbons, mono-, di- and triglycerides, free fatty acids and alcohols are also to be detected[46, 47]. When comparing meibomian and internal tissue lipids a striking difference between them is the presence of branch chains in meibomian lipids[46]. Carbon numbers (the number of carbon atoms in the fatty acid chain) are predominantly even in number for normal and iso-chains while anteiso-chains always have odd carbon numbers. Meibomian lipids also have a much wider

range of chain lengths when compared with tissue lipids[46]. The biosynthesis processes involved in the synthesis of the lipids are probably the cause of such differences[46]. Mass spectrometry (MS) has proven to be an important tool in analysing tear lipids. Oleamide, a fatty acid, phosphocholine (a sphingomyelin), myristamide, palmitamide, stearamide, erucamide (all fatty acid amides) and free fatty acids (myristic, palmitic, stearic and oleic) have all been identified in tear lipids[48, 49] making use of MS. Butovich[50], however, found oleamide to make up an extremely minor component of meibum (below 0.5% of dry meibum weight) and suggested that improper laboratory and collection techniques resulted in previous reports of significant amounts of oleamide being present in tear lipids. Other components of tear lipids detected by MS include: squalene, steroids and steryl esters, wax esters and di- and triacyl glycerols[50]. An apparently new group of lipid compounds in tear lipids have recently been identified using MS. A diacylglycerol-based anionogenic lipid has been detected by the MS technique[51]. Meibomian secretions have also been shown to contain proteinaceous material[52]. The transport of fatty acylglycerols in the circulation involves an entity known as a chylomicron[34]. The transfer of phospholipids from the chylomicron and very low density lipoproteins (VLDL) to high density lipoproteins (HDL) requires the presence of phospholipid transfer protein (PLTP)[53]. PLTP has been isolated in human tears and is thought to play a role in the transfer of meibomian lipids to the surface of the tear layer[54]. Other possible roles that PLTP may play are to scavenge hydrophobic material from the cornea or to scavenge lipids by binding and transporting them into and through the lacrimal drainage system[54].

The statement "The great diversity of lipids found in the meibomian glands and aqueous tears, which are estimated to be present as thousands of individual species, makes it impossible to evaluate, identify and quantify them in one study…"[50] implies that there is still much to be discovered relating to the lipids present in human tears and their functions.

Mucins

Mucin found on the ocular surface is typically described as: "being associated with two sub-layers: an innermost tightly bound glycocalyx layer that is secreted by, and in intimate contact with, the microplicae of the conjunctival and corneal epithelia and an overlying thicker and looser mucous blanket believed to be produced by the conjunctival goblet cells"[8]. The glycocalyx is an extrinsic, carbohydrate-rich surface coat that is found along the apical surfaces of the epithelial cells[55]. The functions of mucin on the eye are considered to be: lubricate and protect the ocular surface, anchor the aqueous tear layer to the ocular surface, protect the epithelium from sheer force damage, drying and bacterial invasion and to provide a hydrophobic scaffold to hold other anti-microbial proteins to the eye[56, 57]. Mucins can be defined as: "glycoproteins, hydrophilic in nature, that have at least 50-80% of their mass as carbohydrate, O-linked to serine and threonine residues present within tandem repeats of amino acids in their protein backbone" (Gendler and Spicer[58] cited in Spurr-Michaud *et al* [9]). The number of genes found, coding for mucin, have increased over the years as the technology to detect them has improved (2000: 9 genes[56], 2001: 14 genes[57], 2004: 17 genes[10], 2007: 20 genes[9]). The genes are named and numbered chronologically in order of their discovery and are indicated as *MUC1, MUC2 etc*[57]. A common characteristic, the presence of a tandemly repeated nucleotide (TR) sequence located in the central part of the gene, is found in all mucin coding genes[57]. Each gene can result in the production of a specific mucin appropriately named MUC1, MUC2 *etc*. Depending on the conformation of the mucin molecule, mucins have been classified as secreted mucins (includes gel-forming and soluble mucins) and membrane-spanning mucins[57, 58]. Secreted mucins are responsible for the rheological properties of mucous and are secreted by the goblet cells while membrane-spanning mucins might interact with intercellular proteins (this does need to be investigated further)[57]. The genes coding for the secreted mucins are all found clustered in the same locus on chromosome 11p15.5[57, 58]. The proteins transcribed from the relevant mucin genes are amongst the largest glycoproteins known with an average molecular weight of approximately 40 MDa[57]. MUC's 1, 3A, 3B, 4, 12, 13, 15, 16, 17 and 20 have been classified as membrane-spanning mucins while MUC's 2, 5AC, 5B, 6, 7, and 19 are classified as secreted mucins[9]. Mucins MUC 1, 2, 4, 5AC and 16 have been found in the tear film[9]. Mucins MUC 4[59] and 16[59, 60] are thought to be particularly important

in maintaining a healthy ocular surface. Recent data suggest three particular membrane-spanning mucins, MUC's 1, 4, and 16, play an important role in dry eye[61]. Also reported in a recent "article in press"[62] is data showing that tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) have dramatic effects on MUC's 1 and 16, two of the major mucins found on the corneal surface. Both TNF-α and INF-γ, inflammatory mediators, are present in dry eye patients and may play a role in ocular surface mucin function.

Closure

One might wonder why it is important for optometry to be aware of the biochemistry of the tear film. Ageing has been shown to be related to alterations in polar as well as neutral lipid profiles of the tear film[63]and as alluded to above, lipids (and age) play a role in the quality of the tear film. Biochemical changes have been shown in the tears and corneas of keratoconic patients, when compared with non-keratoconic patients[64]. Dry eye has been shown to up-regulate at least six different tear proteins while down-regulating at least four[65]. Contact lens wear can also result in changes to tear biochemistry[66]. Changes in meibomian gland lipid secretions have been suggested as being causative in blepharitis[67]. There are numerous instances where an understanding of biochemistry (even a superficial understanding) would enhance optometry's ability to make improved decisions regarding many relevant clinical entities.

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