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Membrane-Associated Mucins of the Human Ocular Surface in Health and Disease

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Abstract

Mucins are a family of high molecular weight, heavily-glycosylated proteins produced by wet epithelial tissues, including the ocular surface epithelia. Densely-packed O-linked glycan chains added post-translationally confer the biophysical properties of hydration, lubrication, anti-adhesion and repulsion. Membrane-associated mucins (MAMs) are the distinguishing components of the mucosal glycocalyx. At the ocular surface, MAMs maintain wetness, lubricate the blink, stabilize the tear film, and create a physical barrier to the outside world. In addition, it is increasingly appreciated that MAMs function as cell surface receptors that transduce information from the outside to the inside of the cell. Recently, our team published a comprehensive review/perspectives article for molecular scientists on ocular surface MAMs, including previously unpublished data and analyses on two new genes *MUC21* and *MUC22,* new MAM functions and new biological roles, comparing human and mouse (PMID: 31493487). The current article is a refocus for the audience of *The Ocular Surface.* First, we update the gene and protein information in a more concise form, and include a new section on glycosylation. Next, we discuss biological roles, with some new sections and further updating from our previous review. Finally, we provide a new chapter on MAM involvement in ocular surface disease. We end this with discussion of an emerging mechanism responsible for damage to the epithelia and their mucosal glycocalyces: the unfolded protein response (UPR). The UPR offers a novel target for therapeutic intervention.

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Keywords

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1. INTRODUCTION

Bodily tissues that face the external environment are frequently exposed to abrasion, desiccation and a wide variety of potential pathogens. One of the functions of “covering” or “coating” epithelia is to protect against such external threats, with specific features adapted to the needs of the specific organ. An example is keratinization, which provides the epidermis with a very effective barrier. However, this is a dry barrier composed of dead and desiccated cells that helps to waterproof the skin [1]. In contrast, most coating epithelia require wetness to fulfill their essential functions. Thus, instead of keratins, the wet epithelia are covered by living cells surfaced by mucins [2].

1.1. Basics of Mucin Structure/Function at the Ocular Surface

Mucins are distinguished by the presence of structural module(s) containing numerous serine and threonine residues that serve as sites for O-linked glycosylation. Usually these modules are composed of tandem amino acid repeat units. The densely-packed glycan chains added enzymatically to these sites by post-translational modification confer the biophysical properties of hydration, lubrication, anti-adhesion and repulsion [3].

There are two types of mucins: secreted and membrane-associated. Secreted mucins, produced by specialized goblet cells, can be soluble or gel-forming. The latter assemble into extremely large oligomeric gels through linkage of protein monomers via disulfide bonds [3]. Secreted mucins create a viscous mucous layer over the epithelia of the tracheobronchial, gastrointestinal and reproductive tracts. In contrast, the aqueous and mucin components of the tear film combine to create a single mucoaqueous gel [4, 5]. A thin lipid layer surfaces this gel, serving to reduce evaporation.

This article focuses on the membrane-associated mucins (MAMs)*. of the ocular surface. MAMs are the defining components of the glycocalyx in all mucosal epithelia. At the ocular surface, they emanate from the plasma membrane of the apical epithelial cells [6], maintaining wetness, lubricating the blink and stabilizing the tear film [2, 5, 7]. In addition, they create a physical barrier against noxious substances [6]. They are by far the largest of ocular surface glycoproteins, with polypeptide chains up to 15,000 amino acids in length [8, 9]. The clustering of O-linked oligosaccharide chains creates steric interactions between carbohydrate and peptide, inducing the peptide backbone to adopt an extended conformation [10]. This results in projection of the MAM well above the cell surface, far beyond other

*HUGO nomenclature used for genes and their products. The abbreviations used are: CT: cytoplasmic tail, ED: extracellular domain; ER: endoplasmic reticulum; ERM: ezrin/radixin/moesin; MAM: membrane-associated mucin; MPS: multipurpose contact lens cleaning solutions; NCBI: National Center for Biotechnology Information; SEA: Sperm protein, Enterokinase and Agrin module; SEM: scanning electron microscopy; UPR: unfolded protein response; VNTR: Variable Number of Tandem Repeats; vWD: von Willibrand Domain

membrane-associated proteins. MAMs would be the first cell-associated molecules encountered by invading pathogens, and are thus well-positioned to shield and protect [6].

Besides their lubricating and barrier functions, it is increasingly appreciated that MAMs also serve an important role as cell surface receptors that sense the extracellular environment and transduce signals intracellularly [11]. In various mucosal tissues, MAMs activate or inhibit intracellular signaling cascades that regulate inflammation, cell-cell interactions, differentiation and cell death [3, 12, 13].

The prototypical MAM is structured much like a classic, single-pass transmembrane immune receptor, as shown in Figure 1. A signal peptide at the N-terminus of the precursor polypeptide chain enables membrane insertion, and may be retained in the final product [14]. The mature protein is composed of two subunits arising from intracellular cleavage, that remain strongly associated via non-covalent interactions [15]. The small subunit consists of a short extracellular region, a single-pass transmembrane domain, and a short cytoplasmic tail (CT). The large subunit, together with the extracellular portion of the small subunit, comprise the extracellular domain (ED).

The tandem amino acid repeat module is located on the ED. The number of tandem repeats for a specific MAM can vary considerably among individuals within a population, thus these repeat-containing module(s) are often called the “VNTR”, for Variable Number of Tandem Repeats [8]. In addition, various conserved sequence modules, such as the Sperm protein, Enterokinase and Agrin module (SEA) and the EGF (epidermal growth factor)-like module, are mixed and matched on the different MAMs.

“Shedding” of the large MAM subunit into extracellular fluids occurs spontaneously or in response to various ligands or stimuli [6]. Ligand binding, shedding of the large subunit, or other external/internal stimuli leads to CT engagement of protein kinases and phosphorylation at specific tyrosine or serine/threonine residues [16]. Phosphorylation initiates signal transduction cascades [11, 17].

1.2. Update on Membrane-Associated Mucins at the Ocular Surface

The Ocular Surface published review/perspectives articles on ocular surface MAMs in 2004 [18], 2010 [19] and 2016 [20]. There have been some more recent reviews on ocular surface mucins that cover both secreted mucins and MAMs [21–24]. Most recently, our team published a comprehensive review/perspectives article for molecular scientists on ocular surface MAMs, including previously unpublished data and analyses on two new genes *MUC21* and *MUC22,* new protein functions, and new biological roles, comparing human and mouse [16]. The current article is a refocus for the audience of *The Ocular Surface.* First, we update the gene and protein information in a more concise form, and include a new section on glycosylation. Next, we discuss biological roles, with some new sections and further updating from our previous publication. Finally, we provide a new chapter on involvement in human disease. We end this with discussion of an emerging mechanism responsible for damage to the epithelia and their mucosal glycocalyces: the unfolded protein response (UPR). The UPR offers a novel target for therapeutic intervention. These areas of focus are summarized in the graphic if Figure 2, in context of the ocular surface.

1.3. Method of Literature Search

A search in PubMed was performed to review literature for the membrane-associated mucins expressed at the ocular surface. A search with the word string “mucin cornea” OR “mucin ocular surface” in PubMed returned 773 articles published from 1957–2021. Of these, 130 articles were reviews. A search with the term “membrane-associated mucin” in PubMed returned 32 review articles published from 1957–2021. In addition to the three articles published in *The Ocular Surface* [18–20], the newer review articles cited above [21–24], and our own article [16], we consider the following as most relevant: [2, 25–32]. Finally, we did a search with the string “MUC1 OR MUC4 OR MUC16 OR MUC20 OR MUC21 OR MUC22” and “ocular surface OR cornea” between the years 2015–2021, turning up 160 articles. More targeted searches were done to find disease associations.

2. MOLECULAR BIOLOGY AND BIOCHEMISTRY

2.1. Genes and Gene Expression

Table 1 lists the twenty-one genes currently classified in the human mucin (MUC) gene group by the HUGO gene nomenclature committee. Eleven of these are expressed by the ocular surface epithelia, goblet cells and associated glands.

Eight of the twenty-one total MUC genes encode secretory mucins. Five of these are expressed at the human ocular surface: *MUC2, MUC5AC, MUC5B, MUC7* and *MUC19.* Only the gel-forming MUC5AC, and very low levels of MUC2, have been detected in the tears (reviewed in [30]).

Thirteen of the twenty-one MUC genes encode MAMs. Twelve of these are “epithelial mucins” expressed by mucosal epithelia. The thirteenth – *EMCN* – is expressed primarily by vascular endothelial cells [33]. Both RNA transcripts and protein products encoded by *MUC1, MUC4, MUC16* and *MUC20* have been detected at the human ocular surface. Protein products encoded by *MUC21* and *MUC22* were localized at the ocular surface for the first time by our team [16].

The expression patterns of the different MAMs are not identical across the human ocular surface. *MUC1* and *MUC16* mRNAs are homogeneously distributed throughout the corneal and conjunctival epithelia [34, 35], while *MUC4* mRNA is predominantly expressed in the conjunctival epithelium [36, 37]. MUC21 and MUC22 proteins have been immunolocalized to the human corneal epithelium; their presence in the conjunctiva has not yet been examined [16].

MUC1, MUC4, MUC16, MUC21 and MUC22 proteins accumulate in cells of the apical layer(s) of the corneal/conjunctival epithelia [16, 34, 35, 37–39]. This fits with their functional role as components of the mucosal glycocalyx elaborated at the ocular surface. In apparent paradox, *MUC1, MUC4* and *MUC16* mRNAs have been localized to cells of all the stratified layers of the corneal/conjunctival epithelia by in situ hybridization [34, 35, 37–39]. One explanation is that the proteins are produced and accumulate throughout the entire epithelia, but mucin glycosylation, and consequently antibody recognition, develops as the cells move up towards the apical layer. An alternative hypothesis supported by the results of

studies in rats, is that newly-synthesized MAM proteins are rapidly degraded, becoming stabilized only in the apical cell layer; [39–41]. The question remains unresolved to date.

MUC1, MUC4, MUC16, MUC21 and *MUC22* are also expressed at ocular surface locations other than the corneal/conjunctival epithelia. Protein products of all these genes have been immunolocalized to acinar and ductal cells of the lacrimal gland [16, 42–44]. Immunoreactive MUC16 protein has been identified in goblet cells of the conjunctiva, associated with the goblet cell mucin granule membrane [45]. *MUC1* is expressed by immune cells that are resident in the ocular surface epithelia, or that infiltrate due to inflammatory or immunological events, including B cells, T cells, monocytes, macrophages and dendritic cells [46–49].

MUC20 protein localization is different from the other MAMs expressed at the ocular surface. It was detected throughout the corneal/conjunctival epithelia, but predominantly within the plasma membrane region of intermediate cell layers rather than the apical layer(s). In the conjunctiva, MUC20 was immunolocalized within the cytoplasm of apical cells, but not in goblet cells [50]. MUC20 expression has not been examined in lacrimal gland.

The shed large subunits of MUC1, MUC4 and MUC16 have been detected in human tear fluid by immunohistochemistry [51]. MUC20 was not found in tears [50]. The presence of MUC21 and MUC22 in tears has not yet been investigated.

2.2. Modular Structure

Table 2 lists the MAMs expressed at the ocular surface, ordered by polypeptide backbone length. These lengths vary dramatically from a maximum of 14,507 amino acids (MUC16) to 484 amino acids (MUC1). MAMs have multiple isoforms, the result of alternative transcription start sites, splice variants, and insertions and deletions. Much of the early interest in MAMs was driven by their pathological roles in cancers, and additional variants may be unique to specific tumors (e.g. MUC1 from a pancreatic tumor [11, 52].

Figure 3 depicts the specific modular architecture of the individual ocular surface MAMs. MUC1, MUC4 and MUC16 are transmembrane proteins that are cleaved during their biosynthesis. MUC1 is cleaved within the SEA module [53], while MUC4 is cleaved within the von Willibrand Domain (vWD) module, carrying a putative cleavage site at the amino acid sequence GDPH. Proteolytic processing of both mucins occurs in the endoplasmic reticulum [54]. MUC16 is cleaved within a juxtamembrane stretch of twelve amino acids located in the ED; this occurs in the Golgi/post-Golgi cellular compartments [55]. Additional MAM cleavages also occur extracellularly and can enhance shedding of the large subunit from the cell surface [56–59].

MUC20 contains hydrophobic regions consistent with plasma membrane association [60], but no alpha-helical transmembrane domain has been identified [16]. Nevertheless, several lines of evidence support the idea that MUC20 binds to cell surfaces [60, 61]. In human ocular surface epithelia, immunoreactive MUC20 was detected predominantly in the cell membrane area of intermediate cell layers and was not detected in human tears, [50]. Thus,

MUC20 appears to be a non-secreted and non-shed MAM that is retained extrinsically at the plasma membrane.

Sequence analysis of human MUC21 and MUC22 predict that they encode transmembrane proteins. It is not known whether they are cleaved during their biosynthesis. A sequence (GSLV) similar to the putative cleavage site associated with the SEA module in MUC1 is present immediately upstream of the putative transmembrane domain in MUC21, however, this sequence is not conserved in MUC22 [62].

As shown in Table 2, the CTs of individual MAMs are dissimilar in length, ranging from 92 amino acids (MUC22) to 22 amino acids (MUC4). Except for the CTs of MUC21 and MUC22, there is no apparent sequence homology or conserved modules [16]. However, their functional convergence is evidenced by the presence of similar protein binding and phosphorylation motifs located within each of the individual CTs [16]. For example, when activated, receptor tyrosine kinase EGFR binds a short motif in the CT of MUC1 and phosphorylates a tyrosine residue [63]. Large subunit shedding stimulates CT phosphorylation and this is enhanced when cells are treated with EGF [64]. Similarly, *in silico* analysis predicts that EGFR also phosphorylates a tyrosine residue on the CT of MUC16 [16].

In fact, EGF signaling appears to be a common theme among the MAMs, accomplished in different ways. The large subunit of MUC4 (but not MUC1 or MUC16) has three EGF-like modules located distal to the cleavage site [65]. The one closest to the transmembrane domain is similar in sequence to the EGF-like domain found in ERBB3, a receptor tyrosine kinase of the EGFR family. Rat Muc4 was shown to interact via this EGF-like module with ERBB2, another member of the EGFR family. The protein-protein interaction induced specific phosphorylation of ERBB2 and led to downstream signaling [66].

As another example of functional convergence, the CTs of MUC1 and MUC16 both connect to the cell's cytoskeleton, but in different ways. MUC1 does this via a binding site for the catenin CTNNB1, which is positioned within the adherens junction that links apical epithelial cells [67]. MUC16 lacks a canonical CTNNB1 binding site, but pull-down experiments suggest that a polybasic amino acid stretch at the proximal end of the CT interacts with ezrin/radixin/moesin (ERM) actin-binding proteins that then interact with the adherens junction [68].

The CTs of MUC1 and MUC16 can also migrate to the nucleus, often initiated by shedding of the large subunit [69–71]. This behavior adds to the possible signal transduction roles by placing the CTs in proximity to transcription factors.

2.3. Glycosylation

Analysis of the carbohydrate content of human ocular surface mucins has revealed that approximately 55% of the mucin mass is carbohydrate [72]. The glycan moieties of MAMs may vary depending on the mucin type, the site of mucin expression, and the physiological or pathological conditions [73]. Glycan structures of ocular surface mucins also display differences among different mammalian species [74].

The synthesis of O-linked glycans is initiated in the Golgi apparatus by the addition of N-acetylgalactosamine to the hydroxyl groups of serine (Ser) or threonine (Thr) amino acids by a group of polypeptide N-acetylgalactosaminyltransferases. The formation of this structure, referred to as the Tn antigen, can be followed by the addition of other carbohydrates such as galactose and N-acetylglucosamine to create core structures, and by the terminal addition of sialic acid, fucose or sulfate. The structures of O-glycan chains found at the ocular surface are relatively simple compared to O-linked glycans in other mucosal secretions, such as the intestinal and respiratory tracts [19]. They are mostly comprised of core 1 which is formed by the addition of galactose in a beta1–3 linkage to N-acetylgalactosamine [75, 76]. The O-linked glycans play an important role in determining the biophysical properties of the MAMs, as well as their extended conformation.

N-linked glycans are also present in the protein backbone of the MAMs. N-glycosylation starts in the endoplasmic reticulum by the transfer of a dolichol oligosaccharide precursor to asparagine (Asp) residues on nascent proteins. At the ocular surface, the N-linked glycans consist primarily of complex-type structures with N-acetyllactosamine. They are not as abundant as the O-linked glycans but play important roles in supporting the stability and function of the mucins [77].

Several biologically important lectins bind the carbohydrate side-chains of MAMs. N-acetyllactosamine, is a preferred ligand for the galectin LGALS3 (galectin-3) [78]. Galectins are involved in barrier function (next chapter), and galectins, as well as selectins and siglecs (sialic acid-binding immunoglobulin-type lectins) are involved in immune responses and immunoregulation [79].

3. BIOLOGICAL ROLES

3.1. Barrier Function

Ocular surface epithelia are continually renewed by division of basal cells at the epithelial basement membrane. As the cells are displaced upwards, they become increasingly flattened and MAMs accumulate in a polarized manner on cells of the apical layer. This creates the transcellular barrier restricting entry into cells. The apical mucosal epithelial cells also develop tight junctions, creating a paracellular barrier that restricts access to lower cell layers [2]. Here we describe two aspects of transcellular barrier function: rose bengal exclusion and pathogen exclusion.

3.1.1. Rose Bengal Exclusion—The study of ocular surface staining by rose bengal, has been instrumental in defining molecular interactions responsible for ocular surface transcellular barrier function. Rose bengal was once commonly used clinically by ophthalmologists as a tool to evaluate ocular surface damage, especially in dry eye [80]. Studies from the Tseng laboratory [81, 82] were the first to show that healthy cells in monolayer culture take up rose bengal, and this can be blocked by mucins. Later, the development of an *in vitro* model of stratified corneal epithelial cells with mucosal differentiation demonstrated that rose bengal is excluded when cells elaborate a mucosal glycocalyx [83].

MAMs of the ocular surface epithelia are crosslinked by LGALS3 to form an interlocking lattice. Like other members of the galectin family, LGALS3 contains a carbohydrate-binding domain with affinity towards beta-galactosides. Abrogation of interaction between LGALS3 and MAMs results in an increase in rose bengal staining [84].

MUC16 expression is essential for the exclusion of rose bengal [68], as well as both O- and N-linked glycosylation [77, 84]. In contrast, knockdown of MUC1 decreases rose bengal penetrance [85]. This apparent detrimental effect of MUC1 on barrier function was reasoned to be a consequence of the huge difference in size and glycosylated residues compared to MUC16: the large and heavily glycosylated ED of MUC16 dominates over the rest of MAMs, providing a wider surface for MAM-galectin interactions to form a tight structure. The other shorter mucins interspersed in the glycocalyx may contribute to create a more uneven lattice that is less efficient in blocking the passage of substances such as rose bengal [85].

3.1.2. Pathogen Exclusion—Exclusion of pathogens by MAMs has been best studied in relation to MUC1, which has been identified as an adhesion receptor for various pathogenic bacteria (e.g. [86–91]; reviewed in [92]). Rather than a means for the pathogen to enter and infect the cell, binding to MUC1 instead appears to inhibit infection by triggering large subunit shedding [87, 89]. In this way, the large subunit serves as a releasable decoy, promoting bacterial clearance. Consistent with this mechanism, knockdown of MUC1 decreased adherence of *Staphylococcus aureus* in cultures of stratified corneal epithelial cells with mucosal differentiation [68, 85].

If MUC1 limits pathogen infection, then increasing the amount of MUC1 on the cell surface should be beneficial. In support of this idea, overexpression of MUC1 by transfection of cultured epithelial cells reduced *Influenza A* viral infection. Similarly, exogenous addition of a mixture of corneal epithelial transmembrane mucins efficiently prevented the interaction of *Herpes simplex* type 1 viral with a human corneal epithelial cell receptor *in vitro* [93]. Several reports describe elevated expression of MUC1 when cells in culture are challenged by bacterial and viral pathogens (e.g., [88, 94, 95]. This may be a consequence of upregulated expression of inflammatory cytokines. For example, when airway epithelial cells were exposed to *P. aeruginosa,* MUC1 upregulation occurred downstream of TNFA induction [88].

Challenge by *Respiratory Syncytial Virus* also strongly induced *MUC21* and *MUC22* expression [95]. *MUC21* was one of the most upregulated RNAs in bronchoalveolar lavage fluid of children with pneumonia due to severe infections of the atypical bacteria *Mycoplasma pneumoniae* [96]. Expression of *MUC22* was remarkably upregulated by double-stranded RNA in normal human bronchial epithelial cells redifferentiated at the air-liquid interface [97]. These findings suggest that, like MUC1, MUC21 and MUC22 are involved in defense against viral infection.

3.2. Regulation of Inflammation

MAMs regulate the inflammatory response in mucosal epithelial cells via interaction with several different signaling pathways. Here we describe

interaction with pathways regulated by NF-kappaB, glucocorticoids, and toll-like receptors.

3.2.1. NF-kappaB—MUC1 is aberrantly overexpressed by diverse carcinomas and certain hematologic malignancies, conferring anchorage-independent growth and tumorigenicity (reviewed in Bafna et al., 2010). This occurs, at least in part, by constitutive activation of the inflammatory pathway regulated by NF-kappaB [98]. The isolated MUC1 CT is sufficient for this effect, when introduced via DNA expression construct into cells that do not express MUC1. The CT of MUC1 interacts with the IKK complex in the cytoplasm, thus releasing NF-kappaB for migration to the nucleus [99]. In contrast, MUC1 expressed at normal levels on mucosal epithelial cells inhibits inflammation due to pathogen exposure by NF-kappaB inhibition [100, 101]. In this case, interaction of the MUC1 CT with IKK prevented NF-kappaB migration to the nucleus [101]. These different results remain to be fully explained.

MUC1 overexpressed by carcinomas is often found in an altered under-glycosylated form. Differences in the glycosylation state of MUC1 can increase its internalization, change its subcellular distribution [102] and alter its interplay with the NF-kappaB pathway [103, 104]. A decrease in the MUC1/A splice variant was observed in dry eye patients [105]. A related study showed some differences between MUC1/A and /B in NF-kappaB activation [106], however, the consequences for the ocular surface are not clear. A possible question for the future is if disease or environment can alter MAMs splicing or glycosylation patterns and affect NF-kappaB activation.

3.2.2. Glucocorticoids—Glucocorticoids are a class of steroid hormones released in response to stress. They activate the glucocorticoid receptor NR3C1, which translocates to the nucleus and binds to the promoters of a battery of genes, activating transcription. Glucocorticoids also inhibit gene expression regulated by inflammatory cytokines and transcription factors AP-1 and NF-kappaB. As part of the natural feedback mechanism that turns down the inflammatory response, glucocorticoid pharmaceuticals are very effective for treating a wide variety of diseases [107].

Glucocorticoids modulate the expression of different MAMs in different tissues [108, 109], including the ocular surface epithelia [110, 111] via response elements located on their transcriptional promoters. The CT of MUC1 interacts with NR3C1, mediating its translocation to the nucleus [112]. Reduced *MUC1* expression is associated with resistance to glucocorticoid drugs [113–115]. In contrast, elevated *MUC4* expression is associated with resistance to glucocorticoid drugs, due to interaction of the CT of MUC4 with NR3C1, which inhibits its nuclear function [116].

3.2.3. Toll-like Receptors—Toll-like receptors (TLRs) are a family of pattern recognition receptors that respond to danger and pathogen-associated signals, thus triggering an innate immune response through the activation of inflammatory signaling pathways [117]. MAMs have been shown to donw-regulate TLR mediated inflammation. For example, in primary bronchial cells, EGFR phosphorylation of the MUC1 CT results in its association with TLR5, inhibiting the recruitment of MYD88 required for signaling [17]. Exposure of

cells to *P. aeruginosa* also results in MUC1 CT phosphorylation, leading to association with TLR5 and suppression of its signaling [118]. In addition to TLR5, MUC1 can also suppress TLR3-, TLR4-, TLR7- and TLR9-initiated signaling, inhibiting the subsequent activation of NF-kappaB [119] and the NLRP3 inflammasome [120–122]. In a model of corneal epithelial cells in stratified culture with mucosal differentiation, both MUC1 and MUC16 dampen the expression of proinflammatory cytokines after the activation of TLR2 and TLR5 [123].

3.3. Determination of Cell Shape and Desquamation

When the ocular surface is viewed from above by scanning electron microscopy (SEM), the apical epithelial cells of humans and other vertebrates exhibit an intricate pattern of plasma membrane folds called microvillae, microplicae and microridges. MAMs emanate from these processes, which increase the cell surface area and are thought to improve the movement of oxygen, nutrients, and metabolic products across the outer cell membranes [2]. When the magnification is decreased, a contiguous mosaic of polygonal cell shapes with a range of sizes, each having a light, medium, or dark appearance, with the dark reflex cells being the largest [124, 125]. The dark reflex is due to a reduction in the size and number of microprocesses [124, 126]. These cells are thought to be the most mature and are shed fairly rapidly, in a process called desquamation. Complete turnover time of the human corneal epithelium has been estimated to be on the order of 1 week [127].

Mechanisms for desquamation of dead corneocytes from the desiccated superficial layer of the dry epidermis are well-described [128]. In contrast, mechanisms controlling desquamation at the mucosal ocular surface, where the apical cells are wet and still metabolically active, are essentially unknown. It has been suggested that loss of microplicae and the ‘lubricating’ MUC16 may facilitate desquamating cell removal [30]. However, these cells must also detach from the underlying cell layer and actively dissociate themselves from their neighbors to undergo desquamation.

A recent study has causally implicated MAMs in both the elaboration of cell processes at the apical epithelial layer of the ocular surface, and in cell-cell dissociation leading to desquamation [85]. First, the same mosaic of light, medium, and dark cells seen at the ocular surface imaged by SEM was also seen in a surface view of a human corneal whole mount, immunostained with antibodies to MUC1 or MUC16 [85]. The intensity of immunostaining correlated indirectly to the cell surface area, with the largest (and presumably most mature) dark cells staining the least. Thus, the reduction in membrane folds and increase in cell area that occurs with cell maturation was found to correlate with loss of MAMs from the cell surface.

The next experiments were conducted using a model of stratified corneal epithelial cells with mucosal differentiation which express both MUC1 and MUC16 on the apical cells in a polarized manner [129]. Surface microplicae/microridges typical of native apical epithelial cells were observed and immunostaining for MUC16 revealed the same surface pattern of light, medium, and dark cells as seen by SEM [85]. Knockdown of MUC16 reduced surface microplicae and resulted in an increase in surface cell area. Interestingly, MUC1 knockdown did not have this effect. In addition, MUC16, but not MUC1 knockdown decreased tight

junction proteins ZO1 and OCLN, as well as transepithelial resistance, a measure of paracellular barrier integrity [85].

Knockdown of MUC16, but not MUC1, was also associated with disruption of the actin cytoskeleton associated with the tight junctions [85]. It was suggested that the CT of MUC16 may mediate this effect by direct interaction with the actin cytoskeleton [85]. Forces originating from cytoskeletal dynamics are posited to generate membrane curvature for the diverse spherical and tubular structures on the cell surface (e.g. [130–132]), thus the CT of MUC16 might also determine microprojections on the cell surface. However, a very recent study provides evidence that the ED of MUC16 would be sufficient [133].

The prototypical MAM ED closely resembles a “brush”, i.e., a well-studied structure in polymer physics, where polymers are grafted on one end to a surface. Polymer brush theory holds that steric interactions in a densely-crowded brush restrict the number of molecular configurations each polymer can explore, thereby reducing entropy and generating pressure on the anchoring surface. Experimental studies with synthetic polymers have confirmed that the pressures generated by these unstructured macromolecules are sufficient to deform flexible lipid membranes (e.g. [134].

To test these hypotheses, a genetically-encoded library of native, semi-synthetic, and rationally designed mucin polymers of varying size, backbone sequence, and membrane anchorage were generated, including the 42 native tandem repeats of MUC1. When expressed at high levels on epithelial cell surfaces, each of the long-chain mucins triggered a dramatic tubularization of the plasma membrane. More rigid, folded protein constructs of comparable size, such as repeating units of highly stable, epidermal growth factor (EGF)-like modules, did not have such an effect. The tubularization phenomenon was relatively insensitive to the length of the mucin polymer domain, provided that the polymers were expressed on the cell surface at moderate to high densities. The results suggested that plasma-membrane morphologies might be predicted simply by the quantity of mucins or other biopolymers on the cell surface [133], including mucosal epithelial cells of the ocular surface.

3.4. Stabilization of Ion Channels

The shed ectodomain of UMOD (Uromodulin) known as Tamm-Horsfall protein, is the most abundant protein in urine, and mutations in this gene cause human kidney disease [135]. Mice expressing *UMOD* with one of the disease-causing human mutations exhibit less urinary Tamm-Horsfall protein, and they also exhibit hypercalciuria and renal calcium crystals corresponding with reduced immunostaining for the renal calcium channel TRPV5 [136]. In transiently transfected human HEK293 cells, co-expression with *UMOD* or addition of exogenous Tamm-Horsfall protein increased TRPV5 surface currents, reduced TRPV5 endocytosis and increased TRPV5 cell surface expression. This is consistent with a role for Tamm-Horsfall protein in stabilization of TRPV5 [137].

Because of structural similarities between UMOD and MUC1, this mechanism was also investigated, with similar results. Interestingly, urinary MUC1 is also reduced in patients with calcium nephrolithiasis, a common type of kidney stone. Moreover, cell culture studies

revealed that TRPV5 surface expression is also enhanced by binding LGALS3. MUC1 enhancement of TRPV5 surface expression is preceded by LGALS3-dependent crosslinking of O-linked glycans on MUC1 with the N-linked glycan on TRPV5.

Thus, LGALS3 crosslinking of MUC1 with the TRPV5 ion transport channel at the surface of epithelial cells appears to provide a novel mechanism for regulation of function. It seems likely that the MUC1 ED might more broadly enhance surface expression of transient receptor potential (TRP) family ion transport channels by a similar mechanism of crosslinking and maintenance at the cell surface. For example, a large genome-wide association study focused on serum concentrations of cations revealed that the highest association with low serum magnesium levels (hypomagnesemia) was a very common genetic variant of *MUC1* (rs4072037) that adds nine amino acids to the extracellular N-terminus of the protein [138].

The findings in kidney suggest possible parallels to the ocular surface. TRP channels have been identified on the ocular surface epithelia, as well as the corneal afferent nerve endings of the ophthalmic branch of the trigeminal nerve [139]. TRPM8 is a cold-sensing receptor activated in ocular surface nerves after evaporation of the tear film to stimulate lacrimal gland secretion [140]. Nerve TRPV1 is activated by hypertonic challenge, which in turn leads to an increased release of pro-inflammatory cytokines [141]. Dysfunction of these ion channels has been suggested as a possible pathophysiological mechanism in dry eye disease [142]. Whether MUC1 can regulate TRP channel activity at the ocular surface will be an interesting question to investigate.

4. DISEASE INVOLVEMENT

Ocular surface diseases are of diverse etiology, but they share a common pathology of damage to the apical layer of the ocular surface epithelia and their glycocalyx, barrier disruption, and cell loss due to programmed cell death. Damage is initiated by internal factors such as meibomian gland dysfunction, blepharitis, rosacea, and by external factors such as ultraviolet light, allergens, pollution, preservatives in glaucoma medication and chemical warfare agents. Ocular surface disease is also seen in Sjögren's syndrome, mucous membrane pemphigoid, graft-vs-host disease and other autoimmune disorders [143–145]. In this section, we discuss the MAM abnormalities observed in different ocular surface diseases. We then go on to discuss an emerging mechanism responsible for damage to the epithelia and their mucosal glycocalyces that offers a novel target for therapeutic intervention.

4.1. Disease Abnormalities

Ocular surface disease is frequently accompanied by alterations in the mucin component of the tears or the glycocalyx associated with the ocular surface epithelia. Abnormalities in O-glycosylation of MAMS have been identified in many ocular surface disorders where the stability of the tears is compromised (e.g., [18]; reviewed in [19]). Here, we discuss MAM alterations seen in four ocular surface disease with different etiologies: dry eye, contact lens wear pathology, and exposure to allergens and pollution.

4.1.1 Dry Eye—Dry eye is a common disease of the ocular surface with multiple causative factors [146–149]. A reduction in goblet cell density is typically observed. This results in a decrease in secreted mucin and leads to tear dysfunction [22, 24]. Variations in the expression levels and glycosylation pattern of MAMs have also been detected, but no clear pattern has emerged.

In one study on Sjögren's Syndrome patients, no change was observed in the steady state level of mRNAs for *MUC1* or *MUC4* mRNA [150]. In contrast, a second study found increased conjunctival MUC1 protein and mRNA in Sjögren's Syndrome patients, as well as increased immunoreactive protein in the tears [151]. In another study with non-Sjögren's Syndrome aqueous deficient dry eye, *MUC1* and *MUC4* gene expression was decreased [152]. *MUC16* gene expression was found increased as well as its presence in tears of Sjögren's Syndrome patients but not in non-Sjögren's Syndrome patients with dry eye [153]. Conversely, another study found lower MUC16 mRNA in patients with aqueous deficient dry eye [154].

Reduced binding of the monoclonal antibody H185 was observed at the ocular surface of patients with non-Sjögren dry eye as compared to normal [155]. The epitope was later identified as an O-acetylated sialic acid found exclusively on MUC16 [35, 156]. This raised the question (still unanswered) as to whether reduced binding was due to a decrease in *MUC16* gene expression, or altered MUC16 glycosylation patterns. Another sialylated epitope, in this case at MUC1, was found increased in both cornea and conjunctiva of dry eye patients, except for the conjunctiva of severe patients [157]. Studies have been directed to the detection of possible changes in glycosyltransferases. While no changes have been observed in dry eye patients [75, 158], different isoforms of polypeptide N-acetylgalactosaminyltransferases and N-acetylglucosaminyltransferases were found altered during ocular surface keratinization [159, 160]. For the polypeptide, N-acetylgalactosaminyltransferases, a change in local distribution but not in their overall expression has been proposed.

Dry eye is age-related and prevalence in the United States population is estimated at nearly twice as high for women as men [161]. However, there is little published on ocular surface MAMs in dry eye with relation to age or sex. In one study, increased amounts of MUC4 was identified in the lacrimal glands of elderly women who received treatment for dry eye [162].

4.1.2 Contact Lens Wear Pathology—Contact lenses artificially separate the tear film into two compartments, thus, challenging its integrity [163–165]. With some frequency, this leads to development of dry eye signs and symptoms. Contact lens wear can reduce goblet cell density and thus, production of MUC5AC, a sign of dry eye [166, 167]. Studies suggest alterations of the ocular surface glycocalyx in contact lens wearers, as evidenced by lower staining with a fluorescein-labeled lectin [168, 169]. Results regarding changes in the expression of MAMs are less consistent [163]. In one study, significant changes in the expression of MUC1, 4 and 16 in contact lens wearers were found [170]. In another study however, changes in different MAMs were detected depending on time and water content of the contact lens [171].

Clinical staining with vital dyes is observed after exposure to contact lenses soaked in certain multipurpose contact lens cleaning solutions (MPS), a phenomenon that has been called solution-induced corneal staining (SICS) [172]. In the first reports, the appearance was described as a 'classic solution-based toxicity reaction' [173, 174]. MPS characteristically contain a surfactant cleaner such as Tetronic 1107 [175], a biocide such as polyhexamethylene biguanide (which may also have surfactant properties [176]), and a buffering agent such as borate. Electron microscopic studies revealed frank loss of surface epithelial cells in rabbit eyes exposed to a borate-based MPS while wearing a contact lens, while control eyes with contact lens wear only exhibited occasional loss of surface membranes but retention of intact junctional borders [177]. When the ocular surface of human subjects was observed in white light at high resolution after they wore a pair of contact lenses soaked in a commonly used MPS, distinct grey/white superficial opaque "dots" could be clearly seen over the transparent, unstained cornea, of a size broadly consistent with the known diameter of superficial human corneal epithelial cells [172].

Significantly decreased levels of mRNA for MUC1 and MUC16 at the ocular surface were found in patients that used an MPS containing boric acid [178]. The Fini laboratory observed only a small decrease in metabolic activity in cultures of human stratified ocular surface epithelial cells with mucosal differentiation treated with three different MPS, and MAM mRNA levels were not significantly changed. However, shedding of MUC16 large subunits into the cell culture medium was observed, correlated with an increase in rose-bengal staining [179]. Exposure to MPS also increased uptake of *P. aeruginosa* uptake by the corneal epithelium of rabbits [177] and cultures of human stratified ocular surface epithelial cells with mucosal differentiation [179].

4.1.3 Allergy—Allergy is a maladaptive immune response to innocuous environmental antigens. Its prevalence has increased in recent years and it is now the sixth cause of chronic illness in the USA [180]. It affects 20–30% of population in industrialized countries, with 50% of affected individuals exhibiting ocular manifestations [181]. Advances in allergy research have changed the way surface epithelia are viewed. They are no longer considered just a barrier. Rather it is now appreciated that they participate actively in the initiation and regulation of immune responses. Activation of pattern-recognition receptors, such as toll-like receptors, on epithelial cells leads to the release of cytokines, chemokines and antimicrobial peptides that attract and activate innate immune cells [182].

Allergic disorders with ocular involvement include seasonal and perennial allergic conjunctivitis and the more severe vernal keratoconjunctivitis and atopic keratoconjunctivitis [183, 184]. Different studies show modifications in the expression of MAMs in these disorders. *MUC1* and *MUC4* mRNA were elevated in patients with atopic keratoconjunctivitis [185]. In another study of the same group, MUC16 was also found elevated [186]. Similarly, increased MUC1 and MUC4 was observed in patients with atopic keratoconjunctivitis compared to controls, but also when compared to patients with vernal keratoconjunctivitis [187].

Additional knowledge on the MAMs implicated in allergy can be obtained from studies on the airways and other tissues. Bronchial cells from severe asthma patients show a reduced

level of the MUC1 CT compared to healthy individuals and this is related to refractivity to glucocorticoid treatment [114]. MUC1 is decreased in patients with allergic rhinitis [188]. A polymorphism of the recently discovered MUC22 is associated with asthma [189]. Elevated MUC1 was observed in long-term smokers, correlating with higher levels of leukocytes, which suggested that the increased MUC1 had an origin in these immune cells [190].

MUC4 overexpression is linked to asthma [116] and eosinophilic esophagitis [191]. In a study of nasal polyp epithelial cells, TLR2 and TLR4 agonists induced the expression of MUC4 [116]. In another study with nasal cells, beta-glucan induced the expression of MUC4 and MUC5B through TLR4 [192]. Also in these cells, diesel particles induced MUC4 expression [193].

Interestingly, allergic response to pollen was abrogated in Myd88$^{-/-}$ and Tlr4 knockout mice. Given that MAMs inhibit MYD88 binding to toll-like receptors, it might be hypothesized that MAMs act to regulate the allergic reaction [181, 194]. This idea remains to be tested.

4.1.4. Pollution—Pollution is a global issue that has severe consequences for health and is responsible for millions of premature deaths every year (reviewed in [195]). Air pollution can affect the eye, causing multiple symptoms (reviewed in [196]). Pollution and cigarette smoke are considered risk factors for dry eye [197]. However, there is still a lack of knowledge on the specific mechanisms of damage by pollutant agents. A few recent studies have investigated alterations to ocular surface MAMs caused by pollutants.

Emissions from sugarcane burning are known to impact on the respiratory health of sugar estate workers and local populations [198]. In a study of the ocular consequences of this practice in Brazil, researchers found alterations in mucin production in workers but also in the residents of neighboring towns [199]. In volcanic settings, airborne, respirable emissions (ash particles, aerosols, and gases) could potentially affect human health [200]. Exposure of conjunctival cells to volcanic ashes induces oxidative stress, an increase in pro-inflammatory cytokines and a decrease in MUC1 [201].

Urban areas, especially those with high levels of traffic, concentrate important amounts of pollutants. In a study with subjects from cities with different levels of pollution levels, goblet cell hyperplasia was detected [202]. Long exposure to traffic pollution, as it is the case for taxi drivers and traffic controllers, causes ocular symptoms and reduces goblet cell density and MUC5AC expression [203]. Diesel exhaust particles increased the expression of IL6 and decreased viability and proliferation of ocular surface epithelial cells. While levels of MUC1 and MUC16 proteins were reduced in corneal cells, they were increased in conjunctival cells [204].

Another common environmental pollutant is cigarette smoke. Passive exposure to E-cigarette smoke for 30 minutes provoked symptoms of discomfort to the eye and airways [205]. Smokers present higher ocular fluorescein [206] and lissamine green staining [207]. Passive cigarette smoke exposure also increased fluorescein and rose bengal staining, which

was maintained after 24 hours of exposure [208]. It seems likely that this is due to effects on MAMs, however, this must be investigated.

4.2. Mechanisms of Damage and Therapeutic Protection

Water-soluble “vital” dyes, including fluorescein, rose bengal and lissamine green, have long been used as a clinical tool to evaluate ocular surface damage [209]. Thus, it is rather surprising how little we understand about what these dyes tell us about ocular surface damage on the molecular level [210]. For example, we know that rose bengal exclusion reflects an intact mucosal glycocalyx (see section 3.1.1). However, what exactly changes in ocular surface disease to disrupt this barrier and cause dye update is less clear.

Fluorescein is the parent compound from which rose bengal is derived, thus, the two dyes are structurally related [211]. Nevertheless, they differ in cell uptake properties. Living corneal epithelial cells in monolayer culture take up fluorescein in the same way as rose bengal, but at a lower level, requiring visualization under epifluorescent illumination [82]. Fluorescein concentration by individual corneal epithelial cells was first observed at the rabbit ocular surface *in situ* [212]. In later studies, individual cells in the superficial epithelial layers of the human ocular surface damaged by dry eye were observed to take up fluorescein, described as “hyperstaining” [213]. Unlike rose bengal, fluorescein uptake by cells was not blocked by mucins [82].

The phenomenon of SICS has provided an opportunity to gain insight into the mechanisms of vital dye staining. In one study, patients wore a pair of contact lenses soaked in an MPS, then their ocular surface was stained sequentially with rose-bengal and fluorescein. Cell-sized pink spots were observed with rose bengal and the same spots were observed with fluorescein. There were some minor differences. With fluorescein, the dots were larger and less sharply-defined, likely due to the well-known diffusibility of this dye. Additionally, some spots of fluorescein staining were observed that did not stain with rose bengal. However, the results suggests that, even if the two dyes identify different types of damage, both are found in most ocular surface cells exposed to MPS [172].

A recent study used cells in monolayer culture to investigate basic mechanisms of fluorescein uptake in cells subjected to damaging stress [214]. While all cells took up fluorescein at a low level, it was found that a small percentage concentrated dye, appearing as hyperfluorescent. Dye uptake was observed to be an active process, inhibited by reducing the temperature or by killing the cells. Application of damaging stress, including hypo- or hyperosmotic stress, exposure to an ophthalmic preservative, or scratch/alkali “wounding” of the cell monolayer greatly increased the number of hyperfluorescent cells. Stressed cells exhibiting high fluorescence intensity also showed characteristics of early apoptosis, whether in monolayer culture, or in the apical epithelial layer of ex vivo rabbit eyes. It was concluded that fluorescein hyperstaining due to damaging stress is an active process of dye concentration by cells that might be compromised, but are still metabolically active [214].

A second team examined fluorescein uptake after MPS-treatment of cells in monolayer culture, with very similar results and conclusions [215]. In a follow-up study, fluorescein uptake in MPS-treated cells was found not to be associated with early apoptotic markers or

metabolic compromise, suggesting minimal toxicity [216]. The surfactant Tectronic 1107 alone caused the same fluorescein uptake effects as the complete MPS [216]. Surfactants dissolve plasma membrane lipids, but this may result in only limited plasma membrane compromise.

To more accurately model the ocular surface, we took up the question of dye uptake mechanisms using both monolayer cells and stratified cultures of human ocular surface epithelial cells with mucosal differentiation [217]. We chose oxidative stress as the damaging stress for our study, created by exposing cells to tert-butyl hydroperoxide (tBHP). Numerous reports have implicated oxidative stress as important to the pathophysiology of OcS diseases [218–221]. Cells produce reactive oxygen species (ROS) as a by-product of their normal metabolic processes and ROS serve as 2nd messengers for intracellular signaling and the level of these potentially damaging substances is controlled by antioxidant systems of the cell. However, under some conditions, ROS become elevated, overwhelming the normal checks.

As visualized under epifluorescent illumination, fluorescein stained all cells in monolayer culture, with dye concentrated primarily in the nucleus. A mosaic of scattered individual hyperstained cells was observed. Oxidative stress increased the number of cells hyperstained by fluorescein. Rose-bengal dye uptake was essentially the same as fluorescein, with similar numbers of cells showing dye concentration under both unstressed and stressed conditions. Fluorescein dye uptake (as quantified by plate reader) stimulated by oxidative stress was inhibited to 35% when the culture temperature was reduced to ambient, and to 10% when reduced to 4°C. These results were very similar to those of the previous reports, consistent with the idea that vital dye uptake and concentration is an active process of living cells [217].

To characterize cell damage caused by oxidative stress, we probed for both early (ANXA5 binding assay) and late (TUNEL assay) stages of apoptosis. Phosphatidylserine exposed on the outer leaflet of the membrane surface leads to ANXA5 binding. The percentage of cells that bound ANXA5 was substantially increased by oxidative stress. In contrast, only a small number of cells appeared to be in late stage apoptosis was detected by TUNEL assay performed at the same time point, and there was little if any difference between unstressed and stressed cells. Again, these results agree with the previous reports and support the idea that cell damage due to oxidative stress in our model is primarily sublethal, at least at the time point examined [217].

To repair plasma membrane damage and maintain proteostasis, cells activate remodeling processes that involve endocytosis [222–225]. We hypothesized that fluorescein dye might be taken up into endocytic vesicles of individual cells undergoing such repair. When monolayer cultures were subjected to oxidative stress, both endocytosis and dye uptake were stimulated. Stress-stimulated endocytosis was blocked by three different endocytosis inhibitors. Stress-stimulated fluorescein dye uptake was blocked by genistein, an inhibitor of caveolin-mediated endocytosis, and chlorpromazine, an inhibitor of clathrin-mediated endocytosis. It was also blocked by the small molecule dynasore, which is required for both forms of endocytosis [217]. Interestingly, in a study published about the same time as our

own, dynasore was also found to inhibit uptake of fluorescein in MPS-treated cells in monolayer culture [216].

Significantly, when we used stratified cultures with mucosal differentiation, we obtained very different results. We found that oxidative stress did not stimulate uptake of TF (transferrin), a marker for endocytosis, in fact there appeared to be some inhibition. Moreover, dynasore and its more potent analogue dyngo-4a, but not other endocytosis inhibitors, prevented staining with both rose bengal and fluorescein in cells subjected to oxidative stress. These results indicate that the link between vital dye uptake and endocytosis, observed in monolayer cells, does not hold for stratified and differentiated cells, which better model the ocular surface. The fact that dynasore and dyngo-4a inhibit vital dye uptake must thus occur through an alternative mechanism [217].

Our cell culture results were validated using *ex vivo* mouse eyes exposed to tBHP in organ culture. As in the cell culture studies, dynasore blocked vital dye uptake at the mouse ocular surface when treatment was performed at the same time as eyes were stressed. Significantly however, dynasore had no effect when used after stress was applied and the ocular surface was already damaged. Thus, vital dye uptake cannot be dependent on endocytosis [217].

If it does not directly block dye uptake, then how does dynasore work? Using metabolic and cytotoxicity assays, as well as western blotting, we demonstrated that dynasore directly protects both cells and their mucin barrier. We concluded that dynasore does not block dye entry, but instead, prevents ocular surface barrier damage and disruption, thus precluding dye uptake [217].

Although designed to inhibit endocytosis by targeting the classic dynamin DNM2 [226], dynasore has “off-target” effects, some of them mediated by other members of the dynamin family with no role in endocytosis. Inhibition of dynamin family member DRP1 using the selective small molecule inhibitor, mdivi-1, protected cardiomyocytes against oxidative stress by preventing mitochondrial fragmentation and apoptosis [227–229]. However, in a second study to investigate the protective mechanism of dynasore, we found that mdivi-1 did not protect either the ocular surface cells or their mucin barrier against oxidative stress. Moreover, while damage to mitochondria and the plasma membrane could be prevented with the RIPK1 inhibitor necrostatin-1, damage to the glycocalyx still occurred [230].

Oxidative stress has been linked to endoplasmic reticulum (ER) stress, caused by an insufficient capacity to fold newly-synthesized proteins [231]. This launches the “unfolded protein response” (UPR), a protective mechanism that restores homeostasis. In the presence of misfolded proteins, the molecular chaperone GRP78 releases UPR sensors ATF6, IRE1 and PERK. Each produces a transcription factor launching gene expression programs that result in increased protein-folding capacity and decreased protein load. However, if stress is excessive or chronic, UPR activation is circuited to an alternate PERK branch involving transcription factor CHOP. This initiates programmed cell death [232].

Emerging evidence links ER stress and the UPR with ocular surface disease. UPR activation in the conjunctival epithelia and goblet cells of mice in experimental and genetic forms of dry eye, and in human Sjögren’s syndrome [233]. In a more recent study from the Argüeso

lab, UPR activation was observed in the conjunctival epithelium of human patients with ocular cicatricial pemphigoid [234]. It was found that treatment of cultured ocular surface epithelial cells with the proinflammatory cytokine TNFA, stimulated expression of UPR markers, as well as MMP9, a key mediator of OcS damage in DE [235]. Importantly, a chemical chaperone attenuated TNFA-stimulated MMP9 expression suggesting that UPR activation is mechanistically linked to inflammation, MMP9 expression and proteolysis [234].

Investigating this idea in our cell culture model, we found that oxidative stress increased mRNA for *sXBP1,* a marker of the IRE1 branch of the UPR, and *CHOP,* a marker of the alternate PERK branch leading to cell death. Oxidative stress also stimulated phosphorylation of eIF2α, the upstream regulator of *CHOP.* Significantly, dynasore selectively inhibited the increase in PERK branch markers [230].

ER stress disrupts mechanisms of Ca^{2+} homeostasis, resulting in release of Ca^{2+} from the ER to the cytosol and to the mitochondria [236, 237], along with UPR activation [238]. In addition to inhibition of PERK branch markers, we found that dynasore also prevented the increase in intracellular Ca^{2+} due to oxidative stress. The increase in PERK branch markers was further inhibited when cells were treated with the cell permeable Ca2+ chelator, BAPTA-AM [230].

We conclude that dynasore protects cells by inhibiting the intracellular Ca^{2+} increase responsible, at least in part, for PERK-CHOP pathway activation in cultures of ocular surface epithelial cells subjected to oxidative stress. Whether the same mechanisms are responsible for damage to the mucin barrier subjected to oxidative stress, and its protection by dynasore, is not known yet, but we continue to investigate.

5. SUMMARY AND FUTURE DIRECTIONS

Herein we described a subset of MAM genes expressed at the ocular surface: *MUC1, MUC4, MUC16, MUC21, MUC22.* Their protein products, heavily modified by addition of O-linked glycan moieties, accumulate at the apical layer of the epithelia covering the cornea and conjunctiva. There, the highly-glycosylated EDs of these MAMs interact with lectins to form the mucosal glycocalyx. These genes are also expressed by conjunctival goblet cells and lacrimal gland. The large subunits of MAMs resulting from cleavage within the ED can be shed into the tears, where they contribute, together with secreted mucin MUC5AC, to formation of a mucoaqueous gel.

It is well-recognized that MAMs, based on their biophysical properties, provide for a stable and transparent refractive surface, maintaining hydration and lubrication, protecting against external challenges and pathogens. However, as discussed herein, it is increasingly appreciated that MAMs play an important role as cell surface receptors that sense the extracellular environment and transduce signals intracellularly. It has been stated that the next big frontier in the MAM field is to expand our knowledge of their function in intracellular signaling [13]. This would be especially important to investigate in relation to disease roles. One that we find most interesting is allergy. As we noted above, allergic

response to pollen is abrogated in Myd88 and Tlr4 knockout mice. Given that MAMs inhibit MYD88 binding to toll-like receptors, we hypothesize that MAMs act to regulate the allergic reaction. This mechanism could also be responsible for ocular surface damage due to dry eye. This will be a very interesting area for study in the immediate future. Pollution promotes the development of both allergy and dry eye, and how this interaction will be another important area to study.

We are also following up on the role of the UPR in damage to mucosal epithelial cells and their glycocalyx and the therapeutic potential of dynasore analogues. The generality of this mechanism to ocular surface disease of diverse etiologies will be important to ascertain. If dynasore analogues are to be used therapeutically, it will also be important to determine their molecular target(s).

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The VNTR (Variable Number Tandem Repeats) modules and regions of unique sequence that are serine and threonine rich and heavily O-glycosylated (O-glyc Regions) are shown with the O-linked glycan chains depicted as “brushes”.

- MUC1: The VNTR module is comprised of tandem repeats of 20 amino acids long, with 25 to 125 repetitions. Flanking the VNTR are two O-glyc Regions.
- MUC4: The VNTR module is comprised of tandem repeats of 16 amino acids long, with 145 to 395 repetitions. Proximal to the VNTR is an Imperfect Repeats module, also heavily-O-glycosylated.
- MUC16: The VNTR module contains long, only partially-conserved tandem repeats of 156 amino acids, with 10 to 60 repetitions. Proximal to the VNTR is a long O-glyc Region of unique sequence (12,070 amino acids) that is also heavily O-glycosylated.
- MUC20: The VNTR module contains tandem repeats of 19 amino acids, with 3–12 repetitions.
- MUC21: The VNTR module contains tandem repeats of 15 amino acid, with 32 imperfect repetitions.
- MUC22: The VNTR module contains tandem repeats of 10 amino acid, with 124 imperfect repetitions.

Other conserved modules are indicated with various shapes defined in the glossary on the graphic and named as in NCBI Gene:

- EGF: Cysteine-rich EGF-like (violet long rectangles)
- SEA: Sperm protein, Enterokinase and Agrin (pale pink, long rectangles)
- Cys-Rich: contains many cysteines (crimson octahedrons)
- NIDO: Nidogen (entactin) (navy triangles)
- AMOP: Adhesion-Associated Domain (yellow stretched hexagons)
- VWD: von Willebrand factor type D (turquoise tall rectangles)
- epiglycanin_C: conserved module found in the CTs of MUC21 and MUC22
- GSLV: proposed cleavage site for MUC21 (mirrored in MUC22)

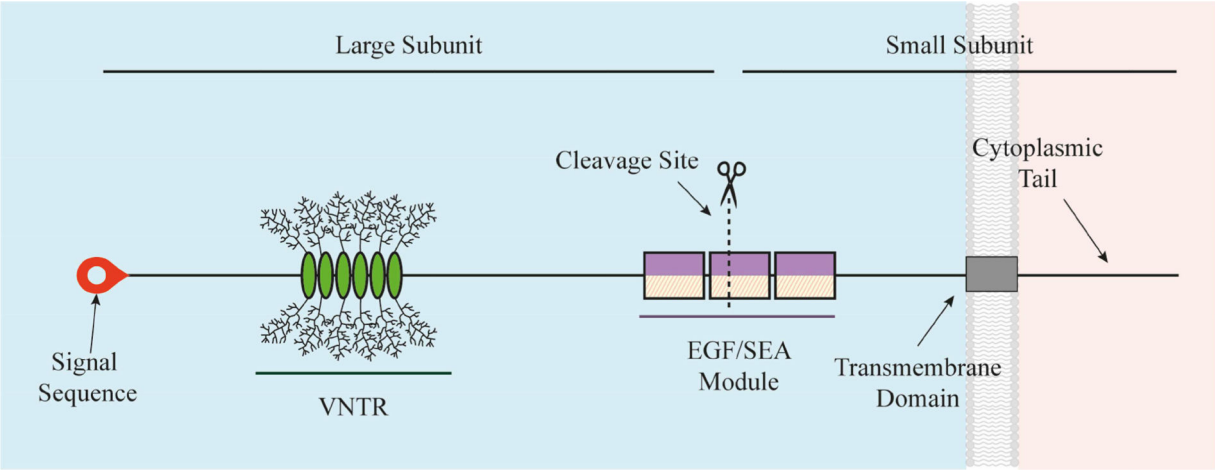

Figure 1.

Molecular Prototype of a Membrane-Associated Mucin.

The graphic depicts a prototypical MAM, the structure of which is like a classic, single-pass transmembrane immune receptor. A signal peptide is found at the N-terminus of the precursor polypeptide chain to enable its membrane insertion; it may be retained in the mature protein [14]. The mature protein is composed of two subunits that self-associate, arising from intracellular cleavage. The large subunit is entirely extracellular and contains the VNTR module with its tandem repeat that are O-glycosylated. The small subunit consists of a short extracellular region, a single-pass transmembrane domain, and a cytoplasmic tail (CT). The large subunit of the MAM, together with the extracellular portion of the small subunit, comprise the extracellular domain (ED). This domain also contains conserved modules such as the Sperm protein, Enterokinase and Agrin module (SEA) and EGF-like modules.

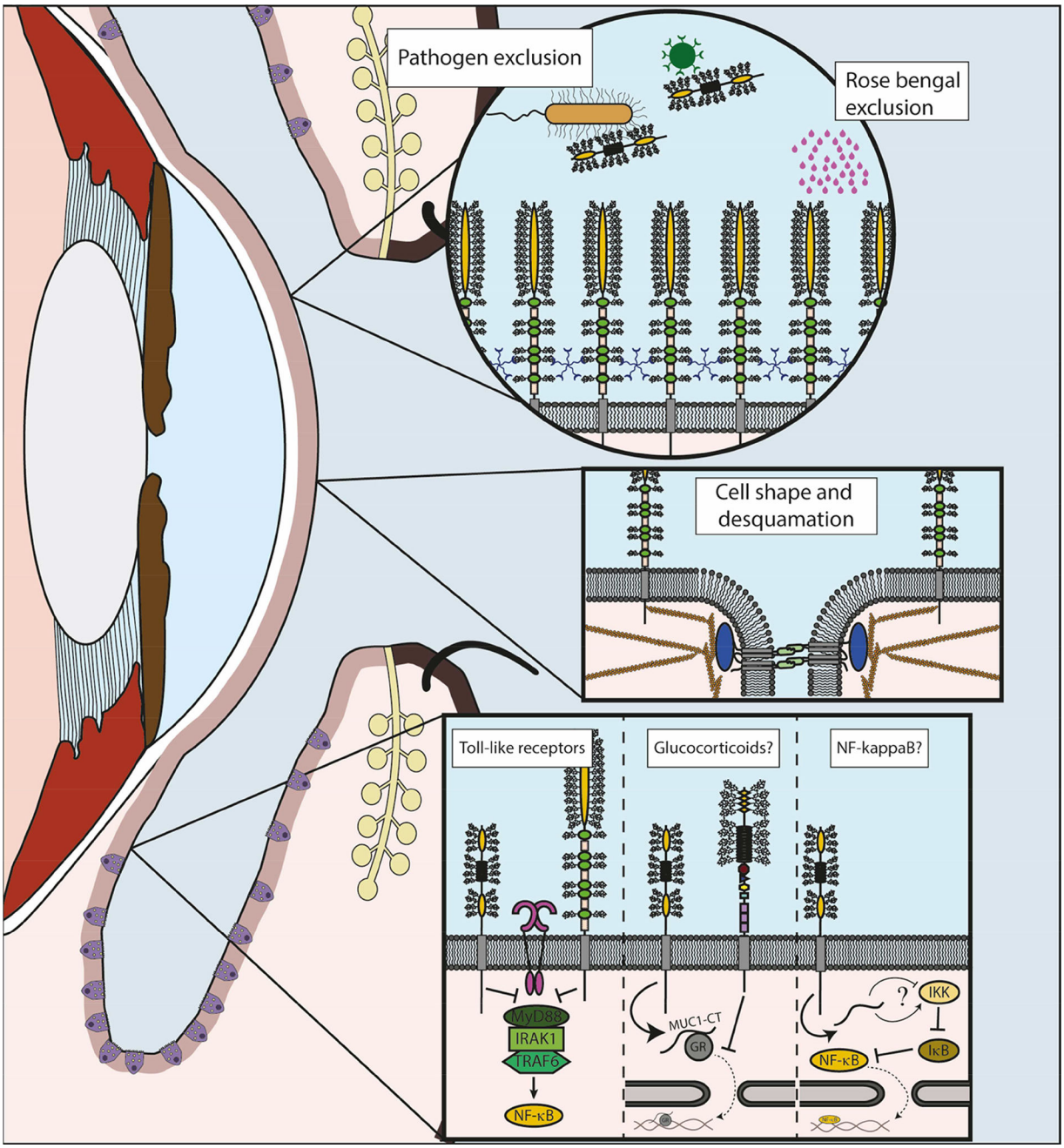

Figure 2.

Functions of Membrane-Associated Mucins at the Human Ocular Surface.

The graphic depicts some of the focus areas of this article.

Left. Sagittal section of the eye and eyelids is represented, with epithelia highlighted in *light pink.* Goblet cells can be observed in the conjunctival epithelium *(purple*). Meibomian glands (*yellow*) can be found at the lids, with their ducts opening at the lid margin.

Top right. Detail of the barrier functions of MAMs. Multiple MUC16 are showed protruding from the plasma membrane as the main elements of the glycocalyx barrier. LGALS3 (galectin-3) pentamers can be found interacting with mucin glycans. This barrier is

responsible of the exclusion of different substances, such as the clinical dye rose bengal. Bacteria and viruses can be excluded also by shed mucins, as it is the case of MUC1, represented here.

Mid right. Detail of the interactions of MAMs with elements of the cytoskeleton. MUC16 participates in the formation of membrane microplicae and tight junctions (represented here with the transmembrane protein occluding and ZO1 in *blue*). Some of these functions have been related to their interaction with actin filaments.

Bottom right. Detail of the immunomodulatory functions of MAMs. A question mark is used to indicate functions that have not been demonstrated in the eye to date. Inhibitory effect on Toll-like receptors (*fuchsia*) has been described for MUC1 *(left*) and MUC16 (*right*) at the ocular surface, reducing the activation of NF-kappaB. Previous studies showed that MUC1 acted by inhibiting recruitment of MyD88. Studies in airways have demonstrated that MUC1-CT interacts with the glucocorticoid receptor (NR3C1), facilitating its migration to the nucleus, while MUC4 (*right*) can inhibit its translocation. Finally, studies in different systems have described interactions between MUC1-CT and different regulatory elements of the NF-kappaB pathway, although the final result of these interactions is not clear.

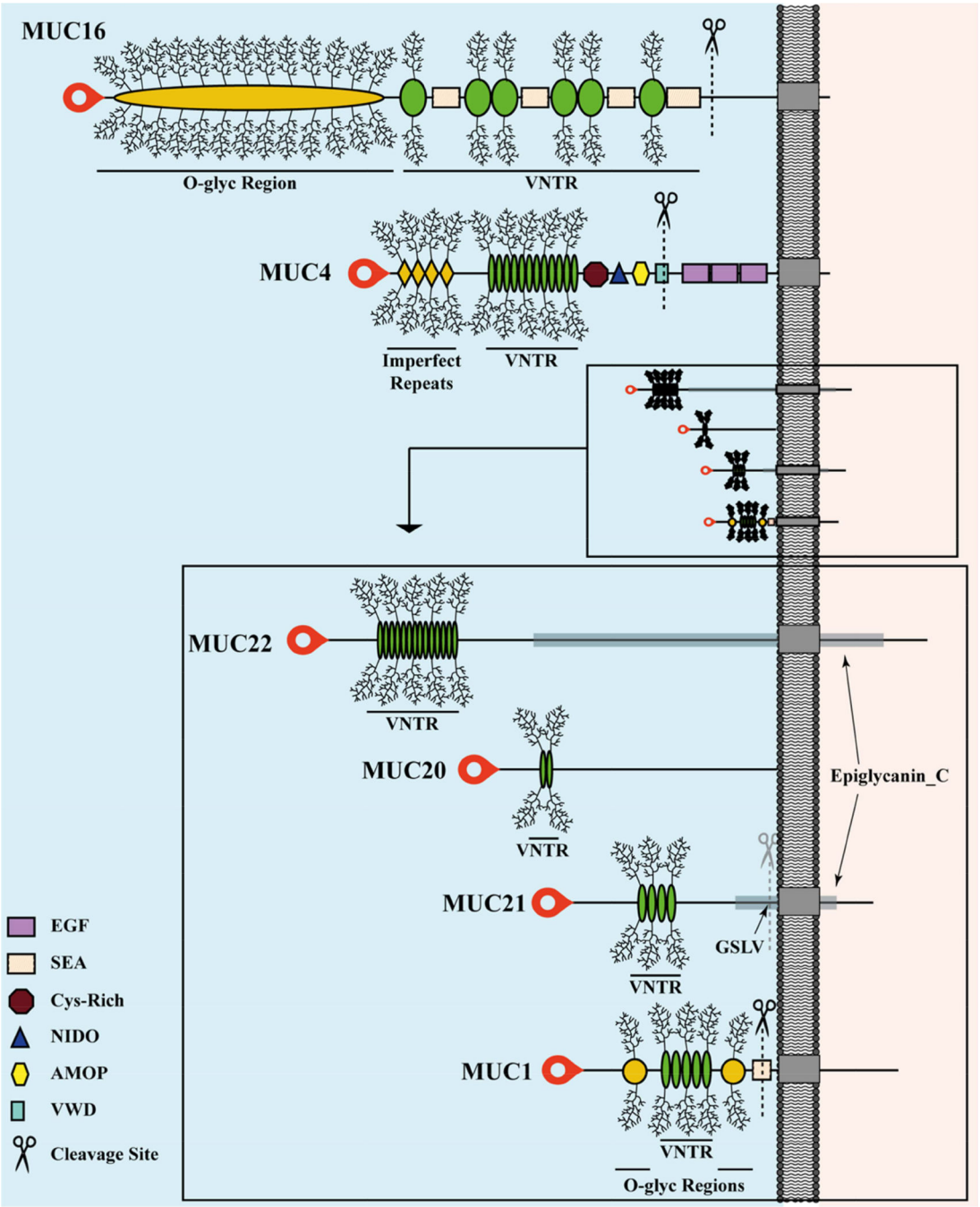

Figure 3.

Modular Architecture of Membrane-Associated Mucins of the Human Ocular Surface. The extended conformation of each extracellular domain (ED) is to the left of the plasma membrane (gray bar) and each cytoplasmic tail (CT) to the right, both drawn to scale. The transmembrane domains are indicated as gray boxes embedded in the plasma membrane. MUC20 has been experimentally determined to associate with the plasma membrane, but has no transmembrane domain, and thus no CT has been identified (discussed in the text). Because of the extreme size differences between the large and small MAMs, an expanded

view of the ED is shown for the small MAMs. Signal peptides are located at the amino-terminus of each protein (red blobs). The cleavage sites in the EDs are indicated by scissors.

Table 1.

Human MUC Gene Family

Gene Symbol	Expressing Tissue(s) at the Ocular Surface	Immunodetected in Tears?
Secreted Mucins (gel-forming)		
MUC2	very low in conjunctival epithelium (RT-PCR only) [171, 239]; not detected in lacrimal gland [162]	detected (low level) [51]
MUC5AC	conjunctival epithelium [36, 50, 171, 239]; goblet cells [36, 51; lacrimal duct goblet cells [43, 162]	detected [51]
MUC5B	conjunctival epithelium (RT-PCR only) [50]; lacrimal gland (RT-PCR only) [42, 162]	not detected [51]
MUC6	not detected in conjunctival epithelium (RT-PCR only) [50, 162]; lacrimal gland (by RT-PCR but not immuno- histochemistry) [162]	N/A
MUC19	corneal and conjunctival epithelia; lacrimal gland [240]	N/A
Secreted Mucins (soluble)		
MUC7	conjunctival epithelium [42, 50, 171]; lacrimal gland (RT-PCR only [42, 162]	not detected [51]
MUC8	not detected in conjunctival epithelium (RT-PCR only) [50]	N/A
OVGP1 (MUC9)	very low in conjunctival epithelium (RT-PCR only) [50]	N/A
Membrane-Associated Mucins		
MUC1	corneal & conjunctival epithelia [38, 50, 171]; lacrimal gland [42, 43, 162]	detected [51]
MUC3A	very low in conjunctival epithelium (RT-PCR only) [50]	N/A
MUC3B	very low in conjunctival epithelium (RT-PCR only) [50]	N/A
MUC4	conjunctival epithelium, corneal epithelium (much less) [36, 37, 50, 171]; lacrimal gland [42] [162]	detected [51]
MUC12	very low in conjunctival epithelium (RT-PCR only) [50]	N/A
MUC13	very low in conjunctival epithelium (RT-PCR only)[50, 171]	N/A
EMCN (MUC14)	expressed primarily by vascular endothelial cells [33]	N/A
MUC15	conjunctival epithelium (RT-PCR only)[50, 171]	N/A
MUC16	corneal & conjunctival epithelia [35, 50, 171]; mucin granules of conjunctival goblet cells [45]; lacrimal gland [44]	detected [51]
MUC17	very low in conjunctival epithelium (RT-PCR only) [50, 171]	N/A
MUC20	corneal & conjunctival epithelia [50]	not detected [50]
MUC21	corneal epithelium [16]	N/A
MUC22	corneal epithelium [16]	N/A

MUC19 is a secreted mucin (gel-forming), a correction from our previous article [16]; MUC12 was previously called MUC11 [30]; N/A: tear presence has not been examined; EMCN [241] and MUC15 [242] lack the typical mucin tandem repeats, however their extracellular domains share the same characteristics, with long extended sequences devoid of secondary structure, densely modified by O-linked glycan chains.

Table 2.

Membrane-Associated Mucins Expressed at the Human Ocular Surface

Symbol	Total amino acids of protein backbone	Predicted mass of protein backbone	Number of Isoforms listed in NCBI Gene	Number of amino acids in cytoplasmic tail
MUC16	14,507	1,519 kDa	14	31
MUC4	7,418	734 kDa	4	22
MUC22	1,786	175 kDa	3	92
MUC20	709	72 kDa	4	N/A*
MUC21	626	60 kDa	3	66
MUC1	484**	50 kDa	20	72

Protein data derived from the NCBI Protein database; listed here is the amino acid number of the longest isoform identified in the database

Estimated molecular weight of the protein backbone mass was computed using:https://web.expasy.org/compute_pi/

*MUC20 is membrane-associated, but does not appear to be transmembrane

**A longer isoform of MUC1, thus far only identified in cancer cells (1255 amino acids) is listed in the UniProtKB database