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Research article

Species variation and spatial differences in mucin expression from corneal epithelial cells

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ABSTRACT

Mucins are large glycoproteins expressed by epithelial cells of both the conjunctiva and cornea, and principle components of the glycocalyx. They are thought to play an important role in determining the interactions between the cornea/conjunctiva and the overlying tear film. The purpose of this study was to characterize the membrane-associated corneal mucin expression pattern from multiple species commonly used in ophthalmic research and drug development to better define the biochemical attributes of the ocular surface. Humans, rhesus macaques and dogs were found to have a very similar pattern of mucin expression, with mucin 16 (MUC16) being the most prevalent mucin transcript. In contrast, the rabbit had a unique mucin expression pattern with all mucin transcripts expressed at relatively similar levels. To determine if there were spatial differences in expression, peripheral and central corneal epithelium were individually isolated and evaluated for mucin expression. In all species examined, MUC1, MUC4 and MUC16 had higher peripheral corneal expression when compared with central, which reached statistical significance in MUC1 (rhesus and dog). The data demonstrated variation in corneal epithelial membrane-associated mucin expression between species, with the rabbit having a distinct expression pattern. These differences may be reflective of the environment, pathogen exposure or tear film dynamics of the respective species. The species differences, as well as regional mucin expression patterns, characterized in this study further define the biochemical composition of the ocular surface and may play an important role in tear film stability.

1. Introduction

The ocular surface is a complex microenvironment comprised of many cellular constituents found on the exposed surfaces of the corneal epithelium, limbus and conjunctiva, which interact with the lid margin and tear film (Yanez-Soto et al., 2014). The integrated interactions of these constituents are responsible for the promotion of a stable tear film and, ultimately, ocular surface health. Mucins are massive glycoproteins expressed by epithelial cells lining all moist surfaces of the body, including the conjunctiva and cornea (Linden et al., 2008). The mucins can be subdivided into two major categories: the membrane-associated mucins and the secreted mucins. The membrane-associated mucins expressed by apical corneal epithelial cells are thought to be the major constituent of the glycocalyx, the dense array of heavily glycosylated proteins extending from the corneal microvilli (Linden et al., 2008). The proposed functions of the membrane-associated mucins include: (1) promotion of water retention, (2) provision of a dense barrier to pathogen invasion or debris, (3) participation in signal transduction (through EGF-like domains), and (4) direct interaction with the actin cytoskeleton (Gipson and Argueso, 2003). Despite significant efforts to characterize the membrane-associated mucins, only a few

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studies have examined the specific contributions of these glycoproteins to ocular surface health (Gipson and Argueso, 2003; Blalock et al., 2007; Govindarajan and Gipson, 2010; Govindarajan et al., 2012; Gipson et al., 2014).

Much of our understanding of these mucins at the ocular surface is derived from experiments with human conjunctival epithelial cells from impression cytology or from experiments using immortalized human corneal and conjunctival epithelial cell lines. The three main membrane-associated mucins highly expressed from the human conjunctival and corneal epithelium include mucin 1 (MUC1), MUC4 and MUC16 (Yanez-Soto et al., 2014). A fourth highly expressed membrane-associate mucin, MUC20, has also been identified in both the cornea and conjunctiva (Woodward and Argueso, 2014). In the conjunctiva, MUC4 is the highest expressed mucin (by quantitative PCR) when compared with MUC1 and MUC16 (Gipson and Argueso, 2003). MUC16 is the largest mucin identified to date, localizes to the microplicae extending from apical corneal epithelial cells and is thought to be the major determinant in corneal barrier function (Perez and Gipson, 2008). A recent study has demonstrated this key role of MUC16 in corneal barrier function through knockdown experiments, where decreased expression of MUC16 lead to increased dye penetrance, increased bacterial invasion and decreased intercellular adhesions (Gipson et al., 2014).

An interesting feature of membrane-associated mucin expression is the spatial variability, as it may provide clues into their physiologic roles at the ocular surface. Regional differences in human MUC4 mRNA expression have been reported with the highest expression of MUC4 in the conjunctiva and limbal epithelia, and a diminishing gradient of expression from peripheral to central cornea (Pflugfelder et al., 2000). It is uncertain if the regional differences in MUC4 expression play a functional role in ocular surface health or stability of the tear film. To date, MUC1 and MUC16 expression has been detected in human cornea and conjunctiva, however, studies examining their regional distribution are lacking.

Despite the thorough characterization of the human corneal mucins, there are no investigations that evaluate the expression of membrane-associated mucins expressed from corneal epithelial cells in species commonly used in ocular surface research and in the development of novel therapeutics, including the rhesus macaque, dog, and rabbit. The comparative approach utilized in this study was designed to fill a knowledge gap with regards to the biochemical composition of the ocular surface from multiple species. Ultimately these data may provide insights into key factors involved in tear film stability as well as help inform the development of novel therapeutics for patients with ocular surface disease, such as dry eye disease.

2. Materials and methods

2.1. Sample procurement

Human corneal buttons were acquired from Saving Sight (St. Louis, MO) stored in Optisol (Bausch & Lomb, Rochester, NY) at 4 °C and used at less than or at approximately 3 weeks postmortem (days: 10, 11, 13, 23, 23). Human donors were selected that lacked historical or observable corneal epithelial pathology. Rhesus macaque and rabbit globes were procured from research animals, which were euthanized for reasons unrelated to the current study. Canine globes were procured from client-owned animals, which had been euthanized for reasons unrelated to our study and deemed unrestricted for use in research at the University of California Davis School of Veterinary Medicine William R. Pritchard Veterinary Medical Teaching Hospital. All fresh globes (rhesus, canine, rabbit) were collected within 2 h of euthanasia.

2.2. Quantitation of mucin mRNA gene transcripts

Corneal epithelium was removed using a #15 Bard Parker (BP) blade under a dissecting microscope, with the debrided zone extending from limbus to limbus (n = 5 globes per species). Total mRNA was isolated from the corneal epithelium using RNeasy Mini Kit (Qiagen Inc, Redwood City, CA) according to manufacturer's instructions and eluted in 30 μl RNAse free water. Isolated total RNA was quantified by UV quantification at 260 nm using a spectrophotometer (Nanodrop ND-1000, ThermoScientific, Wilmington, DE). Human mucin mRNA gene transcripts were quantified using aptamers specific for human MUC1, MUC4, and MUC16 (Table 1, Applied Biosystems/Life Technologies). Ten nanograms of total RNA was reverse transcribed into cDNA and PCR amplified in a StepOne Real-Time PCR System (Applied Biosystems/Life Technologies) with the following parameters: 50 °C for 30 min followed by 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

To evaluate mucin mRNA gene transcripts from rhesus macaques, dogs and rabbits, 0.5–1.0 μg of total RNA was treated with DNase I and reverse transcribed into cDNA according to manufacturer's protocol (Maxima Universal First Strand cDNA Synthesis Kit, ThermoScientific, Wilmington, DE). We identified suspected homologs of mucin genes for rhesus macaque, canine and rabbit transcripts through multiple databases (NCBI, UCSC Genome Bioinformatics, Entrez), using each human mucin mRNA sequence as the template. After homologous sequences were identified, the entire predicted gene sequence was retrieved and all predicted exons were mapped for each mucin. Interexonic PCR primers were designed using MacVector software (MacVector Inc., Cary, NC; Table 1) based on these predicted exons which were tested *in silico* using BLAST searches against the sequence database from the individual species. Low E-values (in most cases <1.0) were considered sufficient in the primer design phase of the experiment. Housekeeping transcripts were selected based upon stability of expression between samples and reported use in previous studies (Brinkhof et al., 2006; Hornsby et al., 2008; Chooi et al., 2013). SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY) was used to amplify cDNA representing 10 ng of total RNA with the following parameters: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by melting curve of 95 °C for 15 s, 60 °C for 1 min with a +0.3 °C/s ramp up to 95 °C for 15 s (Applied Biosystems StepOne Real-Time PCR System, Applied Biosystems). Relative expression was determined by using the $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008), the mean of 5 samples per species were calculated and means were normalized to relative MUC1 mRNA expression. Standard error between the samples was calculated and carried through the normalization of the data to relative MUC1 mRNA expression. Water was substituted for cDNA in nontemplate controls and all qPCR reactions were run in triplicate. To ensure amplification specificity, the PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA), directly sequenced and compared with sequences deposited on the NCBI website (http://www.ncbi.nlm.nih.gov). Due to the variations in mucin gene sequence both within and between species, as well as variations in housekeeping genes used for normalization of data between species, all comparisons were qualitative.

2.3. Evaluation of spatial differences in mucin mRNA expression

The corneal surface from rhesus macaques, dogs or rabbits (n = 6 globes per species) was divided into two geographic regions, the central cornea (defined as a circular region with a diameter approximately half the diameter of the entire cornea, ~25% cornea surface area) and peripheral cornea (rim outside of the central cornea extending to the limbus, ~75% cornea surface area). Using a