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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,

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characterized in this study further define the biochemical composition of the ocular surface and may play an important role in tear film stability.

Keywords

Mucin; Cornea; Ocular surface; Ophthalmology

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 studies have examined the specific contributions of these glyco-proteins 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).

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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

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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, 0.5e-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 $2^{- \ Ct}$ 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 #15 BP blade the peripheral corneal epithelium was debrided separately from the central corneal epithelium, secondly a fresh #15 BP blade was used to debride the central cornea. RNA isolation, cDNA synthesis and quantitative PCR for mucin and housekeeping gene transcripts were performed as previously described. Relative expression was determined using the $2^{- \ Ct}$ method, the mean of 6 samples per species and spatial location was calculated and means were normalized to relative MUC1 mRNA

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expression. Standard error between the samples was calculated and carried through the normalization of the data to relative MUC1 mRNA expression.

2.4. Western bot of MUC16 protein expression

Epithelial cells were debrided from the corneal surface with a #15 BP blade and submerged in 100 μl of 2% sodium dodecyl sulfate (SDS) in 1× phosphate buffered saline (PBS, pH 7.4) supplemented with protease inhibitors (Fisher Scientific, Hampton, NH) on ice. The cells were homogenized, centrifuged at 1000g for 20 min at 4°C to pellet cellular debris and the resulting supernatant was used for the subsequent steps. Protein was quantified using a modified Lowry assay (DC assay, Bio-Rad, Hercules, CA) and bovine serum albumin served as the standard, and analyzed using a plate reader (Synergy, BioTek, Winooski, VT). Total protein (30 μg) was added to 4 × LDS sample buffer (NuPAGE) and separated by agarose gel electrophoresis (0.7% w/v dissolved in 1 × tris-acetate EDTA buffer with 0.1% SDS, SeaKem LE Agarose, Lonza, Switzerland) run at 80 V for 90 min, until the dye front had migrated almost 2/3 of the gel length. The agarose gel was incubated in 1 × SSC buffer for 5 min at room temperature. Protein was transferred from the agarose gel to an activated PVDF membrane (Millipore, Billerica, MA) via capillary transfer overnight at room temperature. The blot was blocked with 1:10 milk diluent/blocking solution (KPL, Gaithersburg, MD) for 1 h at room temperature, and probed with a mouse monoclonal MUC16 antibody (OC125, Abcam, Cambridge, MA) at a 1:500 dilution in PBS-Tween 20 and 1:20 milk diluent/ blocking solution overnight at 4°C. After subsequent washes, the blot was incubated with goat α-mouse secondary antibody (KPL, Gaithersburg, MD) at a dilution of 1:20,000 in PBS-Tween 20 and 1:20 milk diluent/blocking solution for 1 h at room temperature. The protein signal was detected via WesternBright Quantum chemiluminescence (Advansta, Menlo Park, CA) and imaged using ImageQuant 350 imaging system (GE Life Sciences, Pittsburgh, PA).

2.5. Statistical analysis

Statistical analyses were performed using Prism 6 software (GraphPad, La Jolla, CA). Multiple comparisons between spatial differences in mucin mRNA expression were made within species (rhesus macaque, dog, rabbit) and performed using repeated-measures ANOVA and significance between groups was tested with post-hoc Tukey's method. Statistically significant differences (p 0.05) were marked with asterisks in Fig. 2.

3. Results

3.1. Comparison of total corneal mucin expression

To date, very little is known about the expression patterns of membrane-associated mucins of species commonly used in ophthalmic research and novel drug development. In an effort to define the key membrane-associated mucins expressed from corneal epithelial cells, PCR primers were designed to specifically amplify *MUC1*, *MUC4*, and *MUC16* gene transcripts from corneal epithelium in humans, rhesus macaques, dogs and rabbits. Humans, rhesus macaques and dogs exhibited similar patterns of mucin expression, with expression levels being MUC16 > MUC1 > MUC4 (Fig. 1A–C). Relative MUC16 mRNA expression was 3-fold higher in humans and dogs, and 8-fold higher in rhesus macaques when compared with

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the second most abundant mRNA transcript, MUC1. For human, rhesus and canine samples, MUC4 expression was considered the lowest. Interestingly, the rabbit had a very unique pattern of expression with all mucins being expressed at relatively similar levels (Fig. 1D).

3.2. Spatial expression of corneal epithelial mucins

Irrespective of the species investigated, the peripheral corneal epithelium tended to have of higher MUC1, MUC4 and MUC16 mRNA expression when compared with the central cornea epithelium (Fig. 2). In the rhesus macaque, dog and rabbit, MUC1 expression was higher (rhesus: 5-fold, dog: 3-fold, rabbit: 3-fold) in the peripheral cornea, yet statistically significant differences in expression (p 0.05) was found only for the rhesus macaque and dog samples. In addition, the difference in MUC4 expression was higher in the peripheral than central cornea in the rhesus macaque (4-fold), dog (10-fold), and rabbit (1.5-fold). MUC16 had a trend of higher mean expression in the peripheral cornea when compared to central cornea but differences did not reach statistical significance.

3.3. MUC16 protein expression from corneal epithelial cells

MUC16 immunoreactivity was identified in human, rhesus and canine corneal protein extracts, which was negative in the rabbit corneal epithelial extract. (Fig. 3). The lack of a clearly defined of immunoreactive band is consistent with previous reports where mixed glycosylation patterns resulted in variable migration of MUC16 through the gel (Spurr-Michaud et al., 2007). In addition, mucin alleles are known to have variable numbers of tandem repeats within the amino acid sequence, which can lead to the expression of co-dominant mucins with differing sizes and the absence of a distinct immunoreactive band on Western blot. Western blots for MUC1 and MUC4 were performed, which revealed positive immunoreactivity with human corneal protein extracts and an absence of immunoreactivity in the rhesus macaque, canine and rabbit samples (data not shown).

4. Discussion

The current study sought to characterize corneal epithelial mucin expression in multiple species to further our understanding of the native biochemistry of the ocular surface. In human, rhesus macaque and canine samples, MUC16 was the mucin expressed at the highest level when compared to MUC1 and MUC4, whereas the rabbit had relatively equal expression of all three mucins. In addition, spatial differences in mucin expression were also identified when comparing central to peripheral cornea. Corneal epithelial mucins tended to have higher expression in the peripheral cornea when compared to the central cornea; however, this relationship was statistically significant only for MUC1 expression in the dog and rhesus. The presence of MUC16 mRNA transcript expression was validated at the protein level in humans, rhesus macaques and dogs via Western blotting with an antibody that recognizes human MUC16. No immunoreactivity by Western blot was discernable in rabbit corneal epithelial extracts, likely due to variation in primary amino acid sequences, as well as glycosylation patterns. The OC125 antibody was originally used to detect an ovarian cancer antigen (CA125), which has since been identified as MUC16 (Yin and Lloyd, 2001). Subsequent to this initial report, other studies have used the OC125 antibody to detect MUC16 protein expression from human corneal and conjunctival epithelial cells (Argueso et

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al., 2006; Blalock et al., 2007; Gipson et al., 2014). To the best of our knowledge, the exact epitope and glycosylation pattern that it recognizes is unknown. The MUC16 antibody has not been validated for use in other species; however, we interpreted a high molecular weight immunoreactive band, equal in migration to human corneal epithelial extracts, as demonstrating the presence of the MUC16 homolog in the rhesus macaque and dog. The current study is the first to demonstrate species variation, as well as spatial differences, in mucin mRNA transcript expression from corneal epithelial cells.

Aqueous, mucin and lipid are the three main constituents of the tear film and are important for the hydration of cornea, thickness and viscoelasticity of the tear film and reduction in evaporation (Dartt and Willcox, 2013; Hodges and Dartt, 2013). Our definition of the ocular surface follows that of a previous publication (Yanez-Soto et al., 2014) and includes the secreted tear film, as well as the molecules expressed from the corneal/conjunctival epithelial cells that interact with the overlying tear film, to promote a stable tear film. The glycocalyx, composed primarily of mucins, is in direct contact with the overlying tear film, and we speculate that variations in the mucin composition and spatial distribution may contribute to the tear film stability. Rabbits have an incredibly stable tear film with a tear film break-up time of almost 30 min (Wei et al., 2013) and an interblink interval of 10 min (Korb et al., 1998). In contrast, the tear film break-up time in humans is reported to be between eight and 30 s (Wei et al., 2013), and approximately 20 s in dogs (Moore et al., 1987). The important question raised from this observation is, what factors contribute to this increased tear film stability in the rabbit? Our study identified a very different membrane-associated mucin profile in the rabbit when compared with the human, rhesus macaque and dog. Does this expression pattern allow MUC1, MUC4, MUC16 glycoproteins to interact with each other or other components of tear film, leading to a more stable tear film? What is the contribution of each mucin to tear film formation and stability? The answers to these questions are unknown and require further investigation. Ultimately, the role of mucins in tear film stability may provide insight into the development of novel therapeutics designed to stabilize the corneal tear film.

Two previous studies have identified spatial differences in human MUC4 expression with higher expression in the conjunctiva, which decreases toward the central cornea (Inatomi et al., 1996; Pflugfelder et al., 2000). In all species examined in our study, there was a trend for higher expression of corneal epithelial mucins in the peripheral cornea when compared with the central corneal. In light of a previous hypothesis that mucins in the glycocalyx increase the wettability of the ocular surface (Lemp et al., 1970), these spatial differences in mucin expression, as well as species differences, may influence the tear film stability. A lower membrane-associated mucin content in the axial cornea could be the result of increased stress due to blinking and a higher desquamation rate, thus contributing to the observation of the tear film break-up time occurring more frequently in the central cornea (Elliott et al., 1998; Liu et al., 2006). A recent study from our laboratory identified the importance of surface chemical heterogeneity in the "pinning" behavior of human cultured corneal epithelial cells with an over-lying liquid (Yanez-Soto et al., 2015). The understanding of variation in spatial mucin expression, both at the regional and cellular level, provides evidence of the role the mucins play in tear film stability at the ocular surface.

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The rabbit is one of the most common species used in the vision science research and in the development of novel therapeutics; however, based on the results of this study, the predictive value of the rabbit as a model in assessment of tear film dynamics and development of tear film therapeutics should be called into question. Due to differences in mucin expression in the rabbit cornea, its interaction with topical medications used to treat dry eye diseases in the experimental setting may be different. In addition, it is difficult to critically evaluate the effect of topical tear replacements in a species with a highly stable tear film and its interaction with a different biochemical microenvironment at the corneal surface. Our findings also suggest that the biochemical composition of the ocular surface in the rabbit likely provides clues regarding key attributes of the ocular surface that result in promoting tear stability. More studies are required to identify the individual roles and contributions of the corneal mucins.

The current study focused on characterizing the expression patterns of corneal membrane-associated mucins in both clinically and experimentally relevant species, for which there is a knowledge gap regarding characterization of mucin expression. Due to this focus, there are multiple limitations of this study. A relatively small sample size from each species was used to characterize these expression patterns and more samples will be required to confirm these trends in the general population. MUC1, MUC4 and MUC16 were specifically selected due to the extensive literature already known in human corneal membrane-associated mucins. This study represents the first of its kind and future investigations will focus on a more complete characterization of mucin expression, including MUC20 (Woodward and Argueso, 2014), from each of the species. We chose to examine large-eyed animals (humans, rabbits, dogs and primates) as they have the most direct veterinary and human applicability, and we plan to focus future studies on the physiochemistry of the ocular surface in these species. While we recognize there can be changes in the viability and integrity of the corneal epithelium in corneal buttons stored in Optisol (Means et al., 1996), this was not assessed in the study and the data represent a close approximation to the native human cornea mucin expression given the limitations of sample procurement. The detection of mRNA transcripts is an important first step in the characterization of mucin expression in corneal epithelial cells. In other organ systems, such as the gastrointestinal tract (Gustafsson et al., 2013), the mRNA transcript quantitation may not be equivalent to the amount of protein expressed, therefore future studies will focus on detection of mucin protein from corneal epithelial cells.

This study identified species variation in membrane-associated corneal mucin expression, with the rabbit having a unique pattern of expression. The rabbit was unique in that it had equivalent expression levels for all mucins examined whereas for the human, non-human primate and the dog MUC16 > MUC1 > MUC4>. In general, for all species there was either a statistically significant or a trend toward a greater degree of MUC expression at the peripheral cornea compared to the central cornea.

5. Conclusions

Mucins are large glycoproteins found on the ocular surface as both secreted and membrane-associated. They are thought to mediate many of the interactions between the host and its

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environment, as well as contribute to the stability of the tear film. Rabbits were found to have a unique pattern of mucin expression with all mucins expressed to an equivalent degree. This was in contrast with the mucin expression pattern in humans, rhesus macaques and dogs, where MUC16 > MUC1> and MUC4. The peripheral cornea tended to have higher expression levels of mucin mRNA transcripts when compared to the axial cornea, which reached statistical significance in MUC1 (rhesus, dog). These species differences, as well as geographic distribution of the mucins on the corneal surface, may play a significant role in tear film stability.

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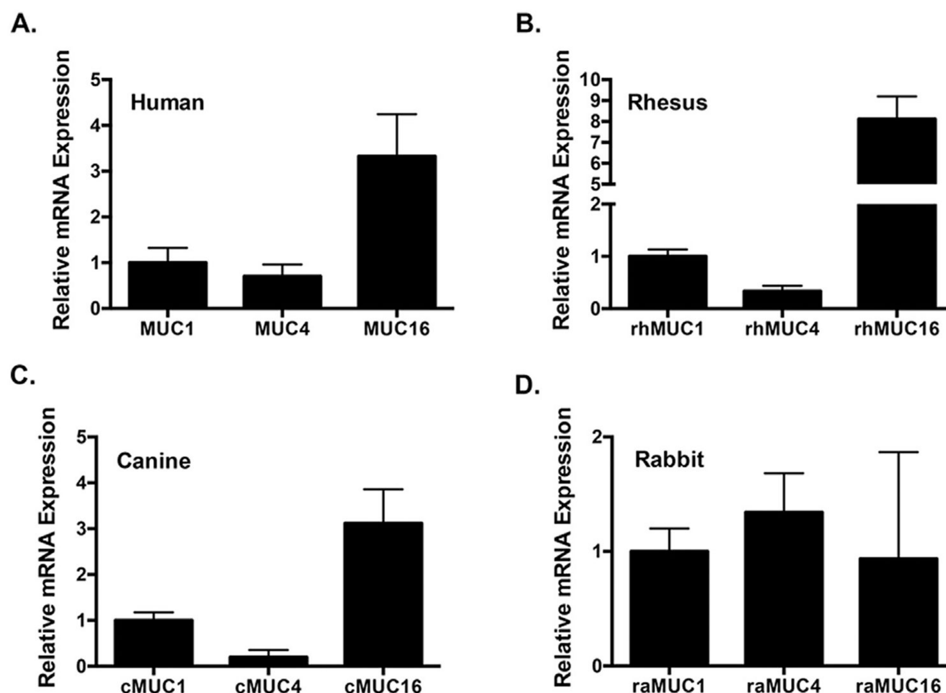

Fig. 1.

Corneal mucin mRNA transcript expression pattern differs between species. Quantitative PCR of mucin mRNA transcript expression in humans (A), rhesus macaques (B), dogs (C) and rabbits (D). Similar patterns of mucin mRNA expression existed in humans, rhesus macaques and rabbits with MUC16 being expressed to the highest levels, followed by MUC1 and MUC4 expression being the lowest detected. The rabbit had a unique pattern of mucin mRNA expression with MUC4 being expressed at the highest level, MUC1 and MUC16 being expressed at lower levels. Data represents relative mucin expression normalized to the respective housekeeping gene per species (Table 1, n = 5 eyes per species from different individuals). Error bars represent standard error of the mean.

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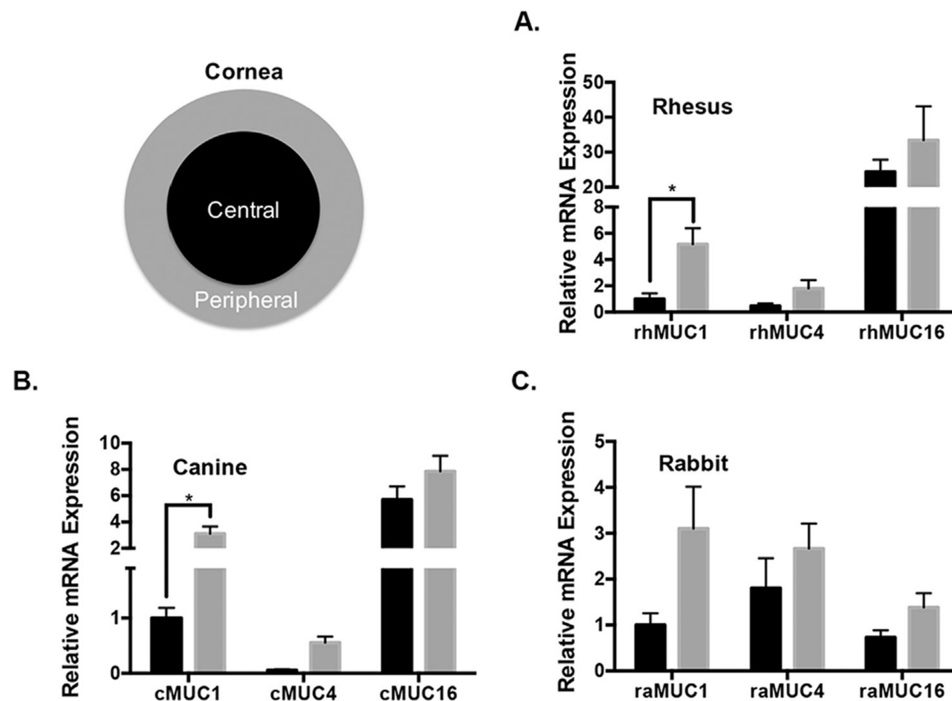

Fig. 2.

The peripheral cornea expresses MUC1, MUC4 and MUC16 at higher levels than the central cornea. Epithelium was debrided from both the peripheral (~75% corneal surface area) and central (~25% corneal surface area) cornea and quantitative PCR was performed. In all species examined (A-rhesus macaque, B-canine, C-rabbit), mucin expression tended to be higher in the peripheral cornea when compared with the central cornea. Data represents relative mucin expression normalized to the respective housekeeping gene per species (Table 1, n = 6 eyes per species from different individuals). Error bars represent standard error of the mean. Asterisk indicates p 0.05.

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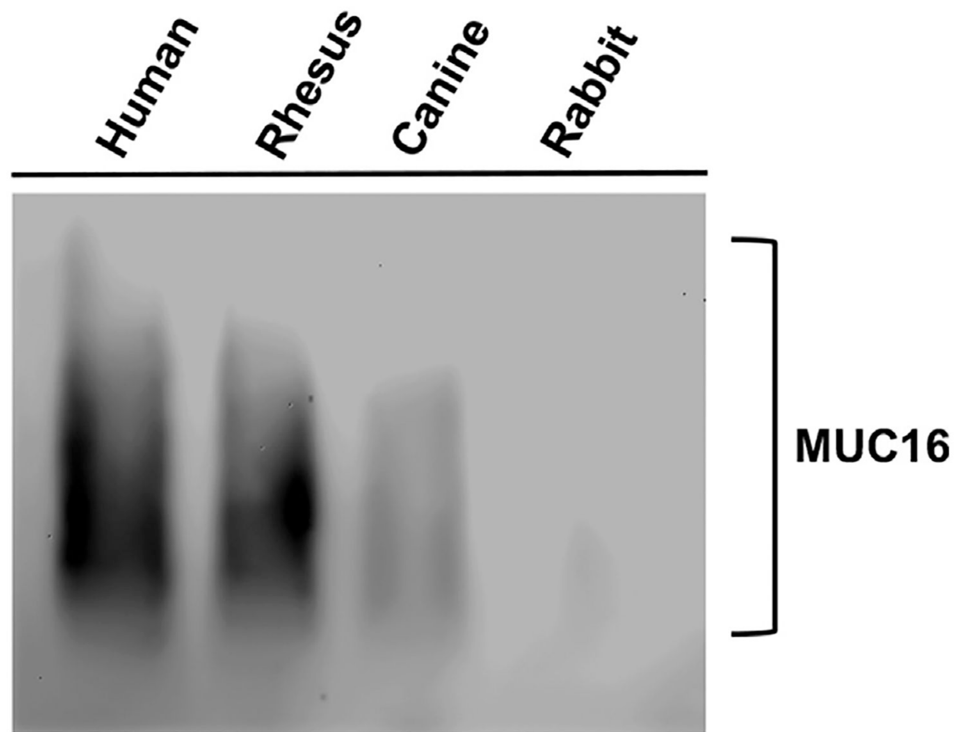

Fig. 3.

MUC16 glycoprotein was detectable in human, rhesus macaque and canine corneal epithelial extracts. Using a human-specific MUC16 antibody (OC125), large high molecular weight protein was detected a human, rhesus macaque and canine corneal epithelial extracts, whereas no protein was detected in rabbit epithelial extracts.

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Table 1

PCR primer sequences and NCBI accession numbers for mucin genes/transcripts. The letter P denotes sequences that are predicted.

mRNA Transcript	Sense	Anti-Sense	NCBI Accession
Human MUC1	Hs001593357_m1		NM_001018016
Human MUC4	Hs00366414_m1		NM_018406
Human MUC16	Hs01065189_m1		NM_024690
18S	Hs99999901_s1		X03205
Rhesus MUC1	5′-CGTAAAACGGAAGCAGCCTCTC-3′	5′-ACAACCAGAACACAGACCAGCAC-3′	XM_001115634 (P)
Rhesus MUC4	5′-ATGTGGTCTTCCAGCCCATCTC-3′	5′-GGTCATAGCCCTTGTAGCCATTG-3′	XM_001102373 (P)
Rhesus MUC16	5′-CTTCACCATCAACAACCTGCG-3′	5′-TCCTCTGGAACAAAGGGCTGAG-3′	XM_001101820 (P)
Rhesus Beta-Actin	5′-TGATGGTGGGCATGGGTCAG-3′	5′-CGTGCTCGATGGGGTACTTCAG-3′	NM_001033084
Canine MUC1	5′-AATCCCAGCAGCAACTACTACCAG-3′	5′-AGTGGCACCATTTCGGAAGG-3′	NM_001194977
Canine MUC4	5′-TCCCCATCAAGCCAGAGAAAG-3′	5′-AAGAGTGGTGAGGTGAAGTCCGTG-3′	XM_545147 (P)
Canine MUC16	5′-TCACCTTGCTGGGAACCTACAC-3′	5′-TGGAGGACAGAGTTGTGGAAGATTC-3′	XM_005633220 (P)
Canine RPS5	5′-GCTCTTTGGGAAATGGAGCAC-3′	5′-GCATCATCATTGAGTTGGTCAGG-3′	XM_533568 (P)
Rabbit MUC1	5′-GCAGAGGAATGTTTCGGCACTG-3′	5′-AGAATCAAGTCCACCACCACGG-3′	XM_008264355 (P)
Rabbit MUC4	5′-TTCAACAACTCCCTCTGTCCCC-3′	5′-AAGTCCACTGTCCTCCTGACGAAC-3′	XM_008266728 (P)
Rabbit MUC16	5′-CTTCACCATCAACAACCTGCG-3′	5′-TCCTCTGGAACAAAGGGCTGAG-3′	XM_008250855 (P)
Rabbit HPRT	5′-TTGTAGCCCTCTGTGTGCTCAAG-3′	5′-CCACCGATTACTTTTATGTCCCC-3′	NM_001105671