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# Human conjunctival goblet cells express the membrane associated mucin MUC16: Localization to mucin granules

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ABSTRACT

MUC16 is an extraordinarily large 22,152 amino acid membrane spanning mucin that has been shown to be present in the glycocalyx of the apical cells of the human cornea and conjunctiva where it interfaces with the tear film. The ectodomain of the molecule has been demonstrated in tears, where it has been presumed to be from surface epithelial cells. Data presented here from multiple assays, including immunohistochemistry, immunoelectron microscopy, *in situ* hybridization, and RT-PCR of RNA isolated from goblet cells isolated by laser capture microdissection, demonstrate that the membrane tethered mucin is also expressed by conjunctival goblet cells both in humans and in mice. The mucin is present in mucin granules and appears to be localized to the mucin granule membrane. Correlation analyses of the amounts of the goblet cell secreted mucin MUC5AC and the amounts of MUC16 and of MUC1 another membrane tethered mucin ectodomain found in human tear samples demonstrated that MUC5AC amounts correlated to the amounts of MUC16 but not to MUC1. These data suggest that goblet cells are a second source of the mucin in tears. The function of the membrane tethered mucin in the mucin granule remains to be determined.



It is well known that goblet cells of the human conjunctiva express and secrete the mucin MUC5AC (Inatomi et al., 1996). This large secretory mucin is packaged within mucin granules that are enveloped by a membrane. In humans, apical cells of the stratified conjunctival and corneal epithelium produce several of a second class of mucins, the membrane spanning or membrane associated mucins. Specifically, the conjunctival epithelium expresses MUC4, MUC16 and in a lesser amount, MUC1 and in central corneal epithelium the predominant membrane mucin is MUC16, with lesser amounts of MUC1 (Gipson and Argueso, 2003). MUC16 is an exceptionally large glycoprotein. At 22,152 amino acids it is the largest of the 19 human mucins. The ectodomain of MUC16 comprises far and away the majority of the molecule, has a series of tandem repeats and is heavily o-glycosylated, particularly in the amino terminal half. It has a short transmembrane domain and cytoplasmic tail of 32 amino acids. (For review of MUC16 structure see Haridas et al. (Haridas et al., 2014), and Govindarajan and Gipson (Govindarajan and Gipson, 2010)). An antibody, termed H185, specific to a glycan on MUC16 (Argueso et al., 2003) was found by immunoelectron microscopy to bind microplicae on the surface of human corneal and conjunctival epithelium, but the antibody was also found to bind to goblet cell mucin packets in human conjunctival epithelium obtained by impression cytology (Danjo et al., 1998). The binding of the antibody to mucin packets in goblet cells, did not rule out the possibility that the antibody was recognizing a similar glycan epitope on another glycoprotein, thus presence of MUC16 in the goblet cell could not be assured from that data.

In the mouse, conjunctival goblet cells express the mouse homologue of MUC5AC, Muc5Ac, and homologue to MUC16, Muc16, which is a much smaller mucin than its human homologue. A recent subtractive microarray analysis of RNA from mouse conjunctival epithelium of mice null for the transcription factor Spdef which lack goblet cells compared to that of wild type mice, demonstrated a highly significant downregulation of Muc16 (RIKEN cDNA 1110008I14) in the Spdef null mice (supplemental data Marko et al.) (Marko et al., 2013). These data suggested that Muc16 might be a goblet cell product in the mouse. The data on binding of human H185 antibody to human goblet cells (Danjo et al., 1998) and the mouse data demonstrating downregulation of Muc16 in conjunctiva lacking goblet cells, led us to hypothesize that conjunctival goblet cells produce the mucin MUC16. We report here, that data

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from several techniques, including immunohistochemical and immunoelectron microscopy using multiple antibodies specific to MUC16, *in situ* hybridization using MUC16 tandem repeat primers, and RT-PCR of laser captured goblet cell RNA conclusively demonstrate that MUC16 is produced by human and mouse conjunctival goblet cells. Subsequently studies were done to determine if the amount of MUC16 in tears correlates to that of the goblet cell mucin MUC5AC present in tears.

Acquisition of human and mouse tissues was done in compliance with Institutional Review Board regulations, informed consent regulations, the tenets of the Declaration of Helsinki and the Schepens Eye Research Institutional Animal Care and Use Committee. Human conjunctival tissues used were those obtained for previous studies (Inatomi et al., 1996), (Danjo et al., 1998), (Kunert et al., 2002). Human tear samples used were obtained for previous studies as reported in Gipson et al. (Gipson et al., 2011).

Immunohistochemical and immunoelectron microscopy analyses were done to localize MUC16 in human conjunctival tissue and on impression cytology samples. To immunohistochemically localize MUC16 either of two monoclonal antibodies both specific to the SEA Module domain (Marcos-Silva et al., 2014), OC125 (Dako Corp., Carpenter, CA) and M11 (NeoMarkers, Fremont, CA), were applied (1:50 dilution in PBS for OC125 and 1:500 in PBS for M11) to frozen sections of conjunctiva (N = 6 tissue samples for each antibody) as previously described (Argueso et al., 2003). Post-embedding immunoelectron microscopy using the monoclonal antibody H185, which recognizes a carbohydrate epitope on MUC16 (Argueso et al., 2003; Argueso and Sumiyoshi, 2006), or monoclonal antibody OC125 was done on LRWhite embedded conjunctival impression cytology samples (N = 10) as previously described (Blalock et al., 2007; Danjo et al., 1998).

*In situ* hybridization analysis was done using $S^{35}$ labeled riboprobes on paraffin sections of human conjunctiva (N = 4 tissue samples) using the methods previously described for *in situ* hybridization of message to MUC5AC (Inatomi et al., 1996). Sections were hybridized with riboprobes overnight at 37 °C and post hybridization washes were at 37 °C. The antisense and sense riboprobes to a tandem repeat region of the MUC16 ectodomain were the same as those employed for fluorescence *in situ* hybridization in human corneal epithelium (Argueso et al., 2003). Oligonucleotide probes were labeled with $^{35}S$ and processed for autoradiography as described (Inatomi et al., 1995, 1996). Hybridization of labeled sense and antisense probes was performed at 37 °C (Argueso et al., 2003). Posthybridiation washes were performed sequentially for 30 min at 37 °C, twice in 2X Saline-Sodium Citrate buffer (SSC), once in 1X SSC and twice in 0.5 X SSC.

Laser capture microscopy was done to harvest clusters of mouse goblet cells, as well as stratified epithelia from both cornea and conjunctiva of frozen sections of the anterior section of 8–12 week old C57Bl/6-129 mixed background mice (6 males, 4 females), in order to assay each epithelial area for presence of full length Muc16 message. RT-qPCR analysis of Muc16 message was done using both primer sets to the Muc16 ectodomain and to the Muc16 cytoplasmic tail. For the ectodomain primers, a pre-validated primer assay set that amplifies a terminal SEA domain in the extracellular domain (ECD) of Muc16 (Qiagen, Valencia, CA) was used. For the cytoplasmic tail assay, a primer set (Forward: AGGGAGACTACCAAGTTCAACG; Reverse: CTGTAACTTCCTCAGGTCCAGG) was designed to amplify mRNA from the cytoplasmic tail (CT) domain of Muc16 using NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/). NCBI BLASTS of primers and translation of amplicons confirmed specificity of each set of primers. Techniques for laser capture and RT-PCR were as previously reported (Marko et al., 2013). RT-PCR experiments were done twice. The endogenous control gene was 18S RNA and relative levels of the mRNA expression were calculated by the ΔΔCT method using the mean ΔCT of the conjunctival goblet cells as the calibrator.

Upon learning that MUC16 was present in human goblet cells, we did Spearman rank correlation analyses (Instat3) of the tear fluid amounts of MUC16, and MUC5AC a secreted goblet cell mucin, as well as with MUC1, another membrane associated mucin present in tears and expressed by stratified epithelium only. Data sets and methods for these analyses were from a previous study of mucins in tears of postmenopausal women with and without dry eye (Gipson et al., 2011).

As demonstrated in Fig. 1A and B, two different monoclonal antibodies, OC125 and M11, both of which recognize SEA modules regions of the protein backbone of the ectodomain of MUC16 (Marcos-Silva et al., 2014) bind to mucin packets of human conjunctival goblet cells. Both antibodies also bind to apical surfaces of the epithelium as have been previously described (Argueso et al., 2003), (Blalock et al., 2007). Immunoelectron microscopic localization of monoclonal antibody H185, which recognizes an o-acetylated sialic acid on MUC16 (Argueso and Sumiyoshi, 2006), to goblet cells obtained by impression cytology demonstrates that MUC16 is present at the periphery of the mucin packets, along the packet membrane (Fig. 1C, D). These data suggest that the membrane mucin is tethered in the mucin packet membrane. Since by light microscopy, the binding of the ectodomain antibodies appears to be present within the mucin packet (Fig. 1A, B) the cytoplasmic tail of MUC16 would appear to face the cytoplasmic surface on the outside of the packet.

A third method documenting the expression of MUC16 by human conjunctival goblet cells is that of *in situ* hybridization. As shown in Fig. 1E and F, $S^{35}$ labeled antisense riboprobes bind heavily to goblet cells of the conjunctival epithelium compared to the control sense probes. Binding of the antisense probes also is seen on the stratified cells of the conjunctiva, in keeping with the demonstrated expression of the mucin by these cells (Argueso et al., 2003).

In the mouse, conjunctival goblet cells occur in basket like clusters (Gipson and Tisdale, 1997). To determine if mouse conjunctival epithelial goblet cells express mRNA to Muc16, clusters of goblet cells were isolated by laser capture microscopy and RNA isolated (Fig. 1G). For comparison, stratified conjunctival and corneal epithelium were also cut from the same sections and RNA from these epithelial regions was also isolated (Fig. 1G). Primers for both the ectodomain and the cytoplasmic tail sequence were used by RT-PCR to determine if full length Muc16 message was present in the three epithelial samples (Fig. 1H). Goblet cells expressed full length Muc16 as demonstrated by presence of comparable amounts of expression of extracellular domain and cytoplasmic tail mRNA. Unlike in the human, mouse corneal epithelium showed no expression of Muc16 and conjunctival epithelium showed a negligible amount.

The presence of ectodomain of MUC16 in tear fluid of humans, which we previously reported, (Blalock et al., 2008, 2007; Spurr-Michaud et al., 2007) was thought to be a result of shedding of MUC16 from the apical surface of the corneal and conjunctival epithelium. Upon determining that MUC16 was produced by goblet cells, we sought to determine if tear levels of the mucin correlated with the goblet cell mucin MUC5AC or with the presence of the ectodomain of another membrane tethered mucin at the ocular surface, MUC1, which is not, as shown by *in situ* hybridization studies, produced by goblet cells (Inatomi et al., 1995). We had previously assayed the amount of MUC5AC, MUC16, and MUC1 in a series of patients with and without dry eye to determine if they correlated with disease (Gipson et al., 2011). Using this data set, we did a correlation test, using Spearman rank correlation in Instat3, between MUC16, MUC1, and MUC5AC and as shown in the table,