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Extending the chinchilla middle ear epithelial model for mucin gene investigation

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Abstract

Objectives—To investigate the expression of recently identified human mucin genes in an in vivo model of the chinchilla middle ear epithelium (CMEE).

Methods—CMEE was harvested, RNA was extracted and primers were designed for RT-PCR to assess for expression of mucin genes Muc6, Muc17 and Muc18. Further sequencing of these genes was also accomplished.

Results—Mucin genes Muc6, Muc17 and Muc18 was assessed and found to be identical to the expression in human and mouse MEE.

Conclusion—This study further characterizes mucin gene expression in the CMEE and provides additional sequence data for chinchilla middle ear genes. The concordance of this gene expression data to that of both the human and mouse models further demonstrates the utility of this animal model in OM investigations.

Keywords

mucin gene; chinchilla; middle ear epithelium; otitis media

Introduction

Otitis media (OM) is a common and costly disease afflicting virtually all children. [1,2] Importantly, in its chronic forms OM is difficult to treat with antimicrobial agents, can be responsible for significant hearing loss and developmental delay and frequently results in the need for surgical intervention. Mucin production in the middle ear (ME) space, in response to the inflammatory events associated with OM, is secreted from ME epithelium (MEE) in order to protect the underlying epithelium and assist with microbial clearance. [3,4] However, these mucins are also the primary elements associated with increased viscosity in ME fluids and when chronically present within the ME are responsible for decreased hearing

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in children. [5,6,7] Because mucins play an integral role in the mechanisms of OM, investigating the expression of mucin genes in this tissue is vital to the understanding of the pathophysiology of OM. The chinchilla (*Chinchilla lanigera*) model has been well defined for both in vivo and ex vivo studies of OM and has historically been the most widely used animal model in studying the pathophysiology of otitis.

Our laboratory has previously characterized the expression of mucin genes in the chinchilla including Muc1, Muc2, Muc4 and Muc5AC and Muc19. [8] In this current investigation we explored the expression of additional mucin genes which have been expressed in epithelial tissues and which we have previously been characterized in the human and mouse. An in vivo chinchilla model was utilized and the expression of Muc6, Muc17 and Muc18 genes was assessed to guide future experimentation in the ME and to generate additional mucin sequencing data in the chinchilla model.

Methods

Animal Donor

Tissues used in these experiments were obtained from mature (6–10 month old), 400–600g mixed breed chinchillas (R&R Chinchilla, Jenera, OH). Animals were treated in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act; the animal use protocol was approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

Prior to tissue harvest, chinchillas were anesthetized with 50 mg/kg ketamine hydrochloride (Phoenix, St. Joseph, MO, USA) and then euthanized with an intracardiac injection of 2 mg pentobarbital (Abbott Laboratories, North Chicago, IL, USA) as approved by the Panel on Euthanasia of the American Veterinary Medical Association. The temporal bone, including tympanic membrane and middle ear cavity, was removed bilaterally. The tympanic membrane and middle ear cavity was examined closely to identify any evidence of infection. Following harvest of the temporal bones, the trachea, stomach and colon were harvested.

RNA Extraction

Immediately after harvest, total ribonucleic acid (RNA) was extracted from the middle ear, trachea, stomach and colon using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A dounce homogenizer was used to homogenize the tissue. DNase digestion was performed using the RQ1 RNase-Free DNase (Promega Madison, WI, USA). The yield and purity was determined by spectrophotometric determination. Purified RNA was stored at –80 °C until RT-PCR analysis.

Primer Design

Primers were designed using published rodent and human sequences and targeted to regions of high homology and sequence complexity. Primer sequences and expected band sizes are detailed in Table 1.

RT-PCR

RNA was reverse-transcribed using SuperscriptIII First Strand Synthesis System with random hexamer primers (Invitrogen Life Technologies, Paisley, UK) and negative control reactions were assembled similarly with the exception of either RNA template or Reverse Transcriptase. PCR reactions contained 0.5uM primer, cDNA template or water (negative control), and Platinum Blue PCR Supermix (Invitrogen Life Technologies, Paisley, UK). Reaction conditions were as follows: 95°C for 5m, followed by 40 cycles of 95°C for 1m,

54-70°C for 1m, 72°C for 1m, and a final elongation step was performed at 72°C for 10m. Primer pairs were initially tested using trachea, stomach and colon cDNA to determine optimal annealing temperature before testing primers on middle ear cDNA. Amplicon was resolved by electrophoresis on 1.5% agarose alongside a DNA marker (500-1,000bp DNA Markers, Cambrex, East Rutherford, NJ) and imaging was performed using Kodak 1D Image Analysis Software Version 3.6 (Eastman Kodak Rochester, NY, USA). Amplicon identity was confirmed by sequence analysis. DNA sequencing was performed by the Protein and Nucleic Acid Facility (Medical College of Wisconsin, Milwaukee, WI) and amplicon sequence identity was determined by BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Muc6 was detected in chinchilla stomach and small intestine (Annealing temperature, TA=67°C; Figure 1 and data not shown) but was not detected in chinchilla middle ear (Figure 1). Chinchilla sequence was 85% homologous to human MUC6. Sequencing data produced a cDNA product of 289 bp (Figure 2).

Muc17 was detected in chinchilla colon and small intestine (TA=63°C; Figure 1 and data not shown) but was not detected in chinchilla middle ear (Figure 1). Chinchilla sequence was 68% homologous to human MUC17. Sequencing data produced a cDNA product of 295 bp (Figure 2).

Muc18 was detected in chinchilla trachea and middle ear (TA=59°C; data not shown and Figure 1). Chinchilla sequence was 83% homologous to human MUC18. Sequencing data produced a cDNA product of 612 bp (Figure 2).

Analysis of full-length human mucin 6, -17, and -18 protein sequences (NP_005952, NP_001035194, and NP_006491, respectively) via SMART (<http://smart.embl-heidelberg.de>) highlighted protein-interaction domains and potential signaling networks (Figure 3). MUC6 was shown to encode three eight-cysteine (C8) domains, three von Willebrand factor (vWF) type D domains, and two epidermal growth factor (EGF)-like domains. MUC17 sequence encoded two EGF-like domains, a SEA domain (domain found in sea urchin sperm protein, enterokinase, agrin), and a transmembrane domain. MUC18 encoded five immunoglobulin (Ig)-like domains and a transmembrane domain. In addition, MUC6 and MUC17 encoded large repetitive proline/serine/threonine-rich domains (mucin domains) which were delineated as regions of low complexity by SMART. Signaling interaction networks were provided by STRING (string.embl.de) through the SMART online interface. Interaction networks provided for MUC17 and MUC18 indicated ten potential signaling partners for each based on evidence collected via text-mining. No protein signaling network was determined for mucin 6.

Discussion

This current investigation focused on 3 mucin genes which have been recently been identified to assess their presence in chinchilla middle ear epithelium. Although MUC6 and MUC17 have not been identified previously in other models examining airway mucin gene expression, the importance of the chinchilla model in the study of OM and differences in mucin gene expression identified in our laboratory between human, mouse and chinchilla models (data submitted for publication), and the importance of these mucin genes in other epithelial tissues prompted their investigation in this model. MUC18 was targeted for examination given its previous identification in our laboratory in both in vivo and in vitro human and mouse models [9].

MUC6 is a secreted, gel-forming mucin encoded by human chromosome 11p15. Similar to other mucins encoded by this region (MUC2, MUC5AC, and MUC5B), MUC6 is characterized by a large mucin-like domain encoding the heavily glycosylated proline-serine-threonine tandem repeat sequence, epidermal growth factor (EGF)-like domains, and cysteine-rich domains which facilitate mucin oligomerization (Figure 3). Polymerization by 11p15 mucins is responsible for the formation of the gel matrix structure which holds beneficial host defense factors close to the cell surface, assists in trapping and clearing foreign particles, and forms a physical barrier against environmental insult [10]. MUC6 glycoprotein is primarily produced in gastric, duodenal, gall bladder, and bile duct epithelial tissues but its highest and best characterized expression is within the gastric epithelium [11-13]. To date, documented expression of MUC6 in airway mucosa is limited to metaplastic tissue (nasal polyps) and expression even in this tissue is debated [10]. The lack of MUC6 expression in chinchilla middle ear reported here corroborates our previous findings in human middle ear as well as similar findings in mouse. The determination that this mucin is absent in each of these models of the middle ear would suggest that further investigation of this mucin as a contributor to respiratory epithelial pathology is not warranted.

MUC17 is a large, monomeric, membrane-tethered mucin belonging to the family of mucin glycoproteins that originate from the human 7q22 gene locus. Similar to other 7q22 mucins (MUC3A/3B and MUC11/12) the large mucin-like domain is followed by two EGF modules flanking a SEA (sea-urchin sperm protein, enterokinase and agrin) domain, a putative transmembrane motif and a short intracytoplasmic tail (Figure 3). During post-translational processing, the SEA domain of the 7q22 mucins is cleaved and the resultant peptides are associated through non-covalent bonds that dissociate under specific conditions at the cell surface [14]. The MUC17 sequence was first reported by researchers investigating MUC3A and was discovered as part of a cDNA chimera transcribed from the MUC3A and MUC17 genes. Although it appears that in humans MUC3A/B and MUC17 are unique genes, a human MUC17 transcription start site has yet to be identified and human MUC17 is currently believed to be orthologous to mouse Muc3 [14]. Given their exposure to the extracellular milieu and potential interaction with intracellular signaling molecules, the transmembrane mucins have been posited to transmit information regarding extracellular conditions internally. Evidence suggests that autoproteolysis of the SEA domain of transmembrane mucins can act as a sensor for mechanical forces, protecting epithelial cells from rupture. Other transmembrane mucins such as MUC1 and MUC4 have been shown to interact with intracellular signaling molecules and MUC17 may possess similar capabilities [14]. Mucins from the 7q22 locus, including MUC17, are all primarily expressed in the gastrointestinal tract. MUC17 is produced throughout the entire intestinal tract demonstrating highest expression in the duodenum [15]. Expression of MUC17 has not been reported in airway mucosa [10], and this current data would suggest that this mucin gene product is also absent from the middle ear and should not be the focus of further airway epithelial investigations.

MUC18/CD146, commonly known as melanoma cell adhesion molecule (MCAM), is considered a mucin based on its post-translational modification by a large number of O-glycosides. MUC18 lacks the proline-serine-threonine tandem repeat domain found in other mucin family proteins, excluding MUC14 and MUC15, and has significant sequence homology to several cell adhesion molecules of the immunoglobulin superfamily (Figure 3). The gene encoding MCAM spans 16 exons on human 11q23 and contains five immunoglobulin-like domains and a cytoplasmic domain marked by several protein kinase recognition motifs [16].

Originally identified as a biomarker for melanoma [17], MCAM is involved in tumor progression and metastasis of a range of cancers [18-21]. In normal adult tissues, MCAM is primarily expressed by vascular endothelium, smooth muscle and lymphocytes [22,23]. In sites outside of the middle ear where a similar respiratory epithelium exists, MCAM expression has been reported in the trachea [24], lung [19], and larynx [25]. MCAM is increased in lung cancer tissue relative to normal tissue [20] and increased expression of MCAM is observed in patients with chronic obstructive pulmonary disease (COPD) compared to smokers or non-smokers without COPD where it is predicted to contribute to airway remodeling leading to obstructive airflow limitation [26]. Our finding of MUC18 expression in MEE of the chinchilla corroborates evidence of MCAM expression in human and mouse MEE [9].

Given the expression of this mucin in MEE and respiratory epithelium future investigations are needed to further elucidate its function in this environment. In the endothelium, the extracellular domain of MCAM mediates cell adhesion, thereby maintaining the integrity of the endothelial monolayer [27], and engagement of this molecule at the cell surface has been shown to initiate a signaling cascade contributing to the formation of focal adhesion plaques and rearrangement of the actin cytoskeleton [28]. MCAM is localized at intercellular boundaries [27]. Activation and subsequent homodimerization of MCAM leads to its interaction with tyrosine kinase p59fyn and phosphorylation of focal adhesion kinase and paxillin, thereby inducing the formation of focal adhesion plaques [28]. The role of MCAM in modulating the dynamics of actin cytoskeleton rearrangement is mediated by its induction of Ca²⁺-dependent phosphorylation of Pyk2 and p130Cas [29,30]. A current area of interest in MCAM research is its importance in lymphocyte targeting and extravasation in sites of inflammation [31]. In the pathogenesis of OM, the potential for MCAM to modulate these effects in MEE warrant further investigation. Finally, SMART (Simple Modular Architecture Research Tool, smart.embl-heidelberg.de) [32,33] and STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, string.embl.de) [34] identify molecular domains of characterized and uncharacterized peptide sequences and predict protein interaction networks based on gene orthology and phylogenetic profile (i.e. conserved occurrence of predicted interacting proteins in different species), as well as co-expression, experimental, database, and text-mining evidence which may be useful in further discerning function and protein-protein interactions to guide further studies planned for our laboratory (Figure 3).

Conclusion

Understanding the mechanisms in which mucin genes regulate the physiology and pathophysiology of MEE will provide a more thorough understanding of the molecular mechanics of the MEE, including disease conditions such as OM, and will be fundamental in providing novel and efficacious interventions. This study further characterizes mucin gene expression in the chinchilla middle ear and provides additional sequence data for chinchilla middle ear genes. This data will be helpful in future investigations examining middle ear pathophysiology and allows for the design of reagents needed in utilizing the chinchilla for these and other investigations. The concordance of this gene expression data to that of both the human and mouse models demonstrates the utility of this animal model in OM investigations.

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Figure 1. Mucin gene expression in chinchilla middle ear epithelium

Agarose gel electrophoresis of amplicon generated from chinchilla cDNA. A housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT), was amplified to control for quality of chinchilla middle ear epithelium (CMEE) cDNA. Chinchilla tissues corresponding to those in which human mucins are known to be expressed (i.e. stomach, colon, and trachea for mucins 6, -17, and -18, respectively) were used to test chinchilla mucin primers.

Chinchilla cDNA	(1)	1315	ATCTCTCTCCAGAGCCACAGCMTCCCGATGAGGNNACTCTCATGGCGGTGTACGAG	1374
HsMUC6, NM_005961	(1315)	ATCTCTCTCCAGAGCCACAGCMTCCCGATGAGGNNACTCTCATGGCGGTGTACGAG		
Chinchilla cDNA	(61)	1375	AAGTCAAGGTACTCACACTCGGNAACCTCCCTGMACTGCTATCATCTACCTGTCCCGAG	1434
HsMUC6, NM_005961	(1372)	AAGTCAAGGTACTCACACTCGGNAACCTCCCTGMACTGCTATCATCTACCTGTCCCGAG		
Chinchilla cDNA	(121)	1435	AGGACAAATCTGTATCTCGAGGATGAGGTATCACCAACCAAGGAGACACCAAGTGGC	1494
HsMUC6, NM_005961	(1430)	AGGACAAATCTGTATCTCGAGGATGAGGTATCACCAACCAAGGAGACACCAAGTGGC		
Chinchilla cDNA	(181)	1495	TGCGGTACAAAGATCGCAICATCAATCTTCAGGCAGACGTCCACCCACCTCCAGATGG	1554
HsMUC6, NM_005961	(1490)	TGCGGTACAAAGATCGCAICATCAATCTTCAGGCAGACGTCCACCCACCTCCAGATGG		
Chinchilla cDNA	(241)	1555	CCACCACTTCGGGCTGGAGCTCGTGGTCCAGCTGGCGCCGCTCTCCAGGTGTCACTCA	1614
HsMUC6, NM_005961	(1550)	CCACCACTTCGGGCTGGAGCTCGTGGTCCAGCTGGCGCCGCTCTCCAGGTGTCACTCA		
Chinchilla cDNA	(301)	1615	CTGTTGGGCCCCAGTTCCAGGCCCAACCCAGAGGCTCTGCGGGCA	1660
HsMUC6, NM_005961	(1610)	CTGTTGGGCCCCAGTTCCAGGCCCAACCCAGAGGCTCTGCGGGCA		
Homology = 85%				
Chinchilla cDNA	(7)	12523	AGTGCCCTCTGMTCCGAACTCTTCTATGGGCCAGGTGTGNAITGATGGTGAAGGCGGT	12582
HsMUC17, NM_001040105	(12523)	AGTGCCCTCTGMTCCGAACTCTTCTATGGGCCAGGTGTGNAITGATGGTGAAGGCGGT		
Chinchilla cDNA	(67)	12583	CGMGNATGGCTGTGCCACACNACGNCN-TCTCTGGCCCTCAAAACTGACTGTGNAAGT	12642
HsMUC17, NM_001040105	(12582)	GACATA--GG----GCCACCGGAGCTATCTCTGCCCAATGGAAGTCACTGTGA--CAOT		
Chinchilla cDNA	(126)	12643	GACCAAGCCAAAGATATCAAGAGTAAAGCTGCAGGCCCTCTTTCAGAAAGAAATTAAGATTT	12702
HsMUC17, NM_001040105	(12635)	GACCAAGCTGTGAAGTTCAACGAGAGCTAAAGAACCTCTTTCAGAAAGAAATTAAGATTT		
Chinchilla cDNA	(186)	12703	CAATTCACATTCCGAGAGCAGATGAAGATAATTTATGACGGAATCCCTGAGTATCAAGG	12762
HsMUC17, NM_001040105	(12695)	CAATTCACATTCCGAGAGCAGATGAAGATAATTTATGATCCGGATCCCTGAGTATCAAGG		
Chinchilla cDNA	(246)	12763	AGTCAACATCACGCAAGCTGAGGCCCGGCAAGTGTGGTGGTGGACCATGA	12810
HsMUC17, NM_001040105	(12755)	AGTCAACATCACGCAAGCTGAGGCCCGGCAAGTGTGGTGGTGGACCATGA		
Homology = 68%				
Chinchilla cDNA	(5)	717	CCAGTGGGAAACACATGAAAGATCTAGGGAAGTCACTGTCCCTGTTTTCTACCCCG	776
HsMUC18, NM_006500	(717)	CCAGTGGGAAACACATGAAAGATCTAGGGAAGTCACTGTCCCTGTTTTCTACCCCG		
Chinchilla cDNA	(65)	777	AGAAAGCTTGTGGTTGGAAAGTGGAGGCTGTGGGAACCTGGAAGGAGAGGAGCCGTGG	836
HsMUC18, NM_006500	(776)	AGAAAGCTTGTGGTTGGAAAGTGGAGGCTGTGGGAACCTGGAAGGAGAGGAGCCGTGG		
Chinchilla cDNA	(125)	837	AATCAAGTGTCTGAGCGATGGCAACCCCTGCGCCCACTTCACTATCAGCAAGAGAAACCC	896
HsMUC18, NM_006500	(836)	AATCAAGTGTCTGAGCGATGGCAACCCCTGCGCCCACTTCACTATCAGCAAGAGAAACCC		
Chinchilla cDNA	(185)	897	CAGCACTGGGGAGATGAGGAACCTGAAACCCCTTGACAAATGGCATCTCGGTCTTGGAGCC	956
HsMUC18, NM_006500	(896)	CAGCACTGGGGAGATGAGGAACCTGAAACCCCTTGACAAATGGCATCTCGGTCTTGGAGCC		
Chinchilla cDNA	(245)	957	TGCCCAAGAGCCGACACAGCGGCTCTACCTAGTGTGAGGCGCTGGACTTGGAGACACAA	1016
HsMUC18, NM_006500	(956)	TGCCCAAGAGCCGACACAGCGGCTCTACCTAGTGTGAGGCGCTGGACTTGGAGACACAA		
Chinchilla cDNA	(305)	1017	ATCACTATCAAGTGAACCCAGAACTCTTGTGGTGAACATATGTGTGGATGTTTCAAGTGA	1076
HsMUC18, NM_006500	(1016)	ATCACTATCAAGTGAACCCAGAACTCTTGTGGTGAACATATGTGTGGATGTTTCAAGTGA		
Chinchilla cDNA	(365)	1077	CCCCGAGCCCCCGAGGCGCAGGAAGGTGCAGCCCTCACATGTAACTGACCGCGGAGAG	1136
HsMUC18, NM_006500	(1076)	TCCCGCAGCCGCTTGAGAGATAGGAAGGCAGGACGCTTACCTGACTGTGAGGCAGAGAG		
Chinchilla cDNA	(425)	1137	TACCAAGCCCTCAATTTCCAGTGGCTGAGAGAAAGACAGGCCAGGTGTGGAAAGAGGG	1196
HsMUC18, NM_006500	(1136)	TACCAAGCCCTCAATTTCCAGTGGCTGAGAGAAAGACAGGCCAGGTGTGGAAAGAGGG		
Chinchilla cDNA	(485)	1197	GTATACGCTTCAGTTAACCAGCTGAAACGGGAGGCCAGGCTGGCTACCGCTCGCTGGG	1256
HsMUC18, NM_006500	(1196)	GTATACGCTTCAGTTAACCAGCTGAAACGGGAGGCCAGGCTGGCTACCGCTCGCTGGG		
Chinchilla cDNA	(545)	1257	ATCTGTGCCCCATATACCCGGCCCTGAACCGCACACAGCTGGTCAACGTGGCCATTTTGG	1316
HsMUC18, NM_006500	(1256)	GTCTGTGCCCCATATACCCGGCCCTGAACCGCACACAGCTGGTCAACGTGGCCATTTTGG		
Chinchilla cDNA	(604)	1317	CCCCCT	
HsMUC18, NM_006500	(1316)	CCCCCT		
Homology = 83%				

Figure 2. Homology of chinchilla and human mucin sequence

Based on NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>.)

Figure 3. Structure, location, and protein interaction network of human mucins-6, -17, and -18

A. Functional domains encoded by human mucin proteins and chromosomal location of human mucin genes based on domain prediction by SMART, smart.embl-heidelberg.de. B. Human mucin protein interaction networks provided by STRING, string.embl.de. Line color indicates type of evidence upon which prediction was based. PDZK1: PDZ domain-containing protein; MUC19: moderately similar to APOMUCIN; GRIN3B: Glutamate [NMDA] receptor subunit 3B; COL6A2: Collagen alpha-2(VI) chain precursor; MAC-2: Galactose-specific lectin 3 (IgE-binding protein); SIP-1: Na(+)/H(+) exchange regulatory cofactor NHE-RF2; PRODH: Proline oxidase; NHERF: Ezrin-radixin-moesin-binding phosphoprotein 50; MUC18: Cell surface glycoprotein MUC18; EGFLAM: EGF-like, fibronectin type III and laminin G domains; TFAP2A: Transcription factor AP-2 alpha; TFAP2B: Transcription factor AP-2 beta; FYN: Proto-oncogene tyrosine-protein kinase; TFAP2D: Transcription factor AP-2 beta-like 1; TFAP2C: Transcription factor AP-2 gamma; TFAP2E: Transcription factor AP-2 epsilon; CD34: Hematopoietic progenitor cell antigen; VWF: von Willebrand factor; ENG: Endoglin; Major glycoprotein of vascular endothelium.

Table 1
Chinchilla primer sequences

Gene	Forward/Reverse Primer	Amplicon Size (bp)
HPRT	GCTCAAGAGCTACTGTAATGAC/ CATATCCAACAACAACTTGTCTG	272
Muc6	CCCTACCGCTTCCACGGCAC/ TTGCCGCAGAGCCCTCTGGT	371
Muc17	CTGCAAGAATGGAGGCACCTGG/ CATGCTCCACCACCACACTGCC	310
Muc18	CCCAGTGGGAACCACATGAA/ CACCCACACCTTCCTCTCCT	639