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Mucin gene expression and mouse middle ear epithelium

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Abstract

Objectives—To investigate the expression of recently identified human mucin genes in an *in vitro* model of cultured mouse middle ear epithelial cells (MMEEC).

Methods—MMEEC were established, RNA was extracted and primers were designed for RT-PCR to assess for expression of mucin genes *Muc1, Muc2, Muc3, Muc4, Muc5AC, Muc5B, Muc6, Muc7, Muc8, Muc9, Muc10, Muc11/12, Muc13, Muc15, Muc16, Muc17, Muc18, Muc19* and *Muc20* expression.

Results—Mucin genes *Muc1, Muc2, Muc3, Muc4, Muc5AC, Muc5B, Muc9, Muc10, Muc13, Muc15, Muc16, Muc18, Muc19* and *Muc20* were identified and expressed in MMEEC. The genes *Muc6, Muc7, Muc8, Muc11/12 and Muc17* were not identified.

Conclusion—Many of the mucin genes that have been recently identified in human MEE and chinchilla MEE are also expressed in MMEEC. There are differences in expression, however, which may have implications in utilizing various animal models for study of middle ear physiology and pathogenesis; specifically as it relates to mucin gene expression.

Keywords

mucin gene; mouse; middle ear epithelium; otitis media

Introduction

Recent efforts to understand the molecular underpinnings of otitis media (OM) have highlighted the importance of mucin gene expression and regulation in the middle ear epithelium (MEE) [1–4]. Composed primarily of respiratory-type epithelium, the ME

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contains numerous mucin secreting cells. As a family of glycoproteins, there are currently 19 uniquely described mucin genes that have been identified. In the epithelium throughout the respiratory tract, including the ME, mucins participate in a number of processes important for the protection and function of the underlying epithelium. These include mechanical protection of the epithelium, mucociliary clearance of pathogens and particulate matter, antigen presentation, and prevention of pathogen adherence and host invasion [1,5]. In the ME, mucins determine ME fluid viscosity and are one of the important components in determining how readily ME fluid is cleared from the ME through the eustachian tube. Although more viscous fluid may provide a greater barrier to pathogen destruction and invasion it also can result in increased difficulty in mucociliary clearance leading to mucostasis and associated hearing loss in children. Each mucin gene product has somewhat different characteristics and, as such, understanding the totality of mucin gene expression in any given experimental model is important for allowing conclusions utilizing that particular model.

The specific aim of this current investigation was to perform a comprehensive investigation of mucin gene expression in mouse ME epithelium from an *in vitro* murine source to characterize the mucin gene expression in this model of MEE and compare these findings to an in vivo mouse ME model. This characterization will provide the foundation for future investigations into the potential role of these various mucins in the physiology and pathophysiology of the MEE and specifically as these mucins relate to otitis media.

Methods

Mouse Middle Ear

Mouse middle ear materials were obtained from 8 adult 129Sv mice. Animal handlings were in accordance with IACUC approved protocol. Following euthanasia, temporal bones were harvested and the middle ear cavity was rinsed with TRIzol reagent (Invitrogen). Total RNA was extracted following the manufacturer's instruction.

Mouse Middle Ear Epithelial Cell Cultures (MMEEC)

Cells used in mouse middle ear cultures were MMEEC whose primary characterization regarding transformation and growth properties have been published [6]. MMEEC were grown in full growth media comprised Ham's F-12K media supplemented with 1.5g/L sodium bicarbonate, 2mM L-glutamine (ATCC), 10ng/ml epidermal growth factor (EGF, R&D Systems), 10mg/ml insulin-transferrin-sodium selenite (Sigma), 2.7g/L glucose, 500ng/ml hydrocortisone, 0.1mM non-essential amino acids (Invitrogen), 4% fetal bovine serum (Invitrogen) and 1% Penicillin/streptomycin (Cambrex). Culture was incubated at 33°C in humidified incubator containing 5% CO_2. Media was changed every 3 days.

Mouse Mucin Primers

Each of the mucin genes studied has been primarily identified in a tissue outside of the middle ear [7]. Primer pairs utilized for measurement of mucin genes in the MMEEC are listed in Table 1. References to primer sequence are listed as PubMed ID number or the probe sequence ID for the published sequence, and GenBank accession number of the sequence used in primer design. Utilizing this approach, primers were readily obtained for mucin genes: *Muc1, Muc2, Muc3, Muc4, Muc5AC, Muc5B, Muc6, Muc9, Muc10, Muc13, Muc15, Muc16, Muc18, Muc19* and *Muc20*. However, the mouse genome would suggest that, in the mouse, there do not exist corresponding genes for *Muc 7, Muc 8, Muc 11/12* and *Muc 17*. To investigate the possibility of expression of genes corresponding to these previously identified human mucin genes primer pairs were developed utilizing human mucin gene sequences. As a designed set of primers failed to demonstrate an expression on

RT-PCR additional steps to ensure the lack of expression were taken. This included designing and testing multiple sets of primer pairs with the potential to amplify non-identified mucin genes and literature search for possible related sequence.

Mucin gene expression

Mucin gene expression analysis was determined by using standard RT-PCR techniques. Total RNA of MMEEC was harvested using the RNeasy Mini Kit (Qiagen). Yield and purity was determined by spectrophotometry. Genomic DNA digestion was performed using RQ1 RNase-Free DNase set (Promega). cDNA was obtained using Superscript III RNase H^- Reverse Transcriptase (Invitrogen), following the manufacturer instruction. Each reverse transcriptase reaction used 2 μg of the previously purified RNA. The cDNA was amplified in 50 μl reaction contained 1.0 unit Platinum Taq DNA polymerase (Invitrogen), 0.2mM each of dNTPs (Invitrogen), 0.2 μM of each primer, and 1 μl of cDNA template using GeneAmp 2400 thermocycler (Perkin-Elmer). A negative control contained every component except the reverse transcriptase was used for each reaction. The PCR reactions were run on 2% agarose gel at 100V. The PCR product was visualized with GelStar (Cambrex).

Reliability of the PCR product generated by the selected primer pairs and identified in the gels was further assessed by exposing the product to appropriate endonuclease digestion listed in Table 1. Ability to “cut” the PCR product into expected size bands was interpreted as confirmation that the generated cDNA sequence did, in fact, represent sequence from the mucin gene in question. In cases where enzymatic digestion was not possible the generated cDNA sequences were sequenced and confirmed by comparison to previously published sequence of the mucin gene in question.

Results

Primer pairs used for each of the mucin gene assessments in MMEEC are listed in Table 1. Mucin genes *Muc1, Muc2, Muc3, Muc4, Muc5AC* and *Muc5B*, which have been previously studied in mouse middle ear, were each assessed and found to be present in both *in vivo* and *in vitro* preparations (Figure 1).

Of the more recently identified mucin genes *Muc9, Muc10, Muc13, Muc15, Muc16, Muc18, Muc19* and *Muc20* were identified.

*Muc*3 is not in the class of recently identified and characterized mucin genes. It has not been discussed previously as an important middle ear epithelial mucin and has not received identification as being present in most studies examining middle ear mucosal mucin gene expression. However, *Muc*3 was also identified in both *in vitro* and *in vivo* specimens in this investigation (Figure 1).

Use of restriction endonucleases (Table 1), to ensure the identified PCR product represented the mucin gene in question, demonstrated appropriate digested products to the corresponding gel bands for mucin genes *Muc1, Muc2, Muc3, Muc4, Muc5AC, Muc5B, Muc9, Muc13, Muc15, Muc16, Muc18, Muc19, and Muc20*. The *Muc10* cDNA scarcely generated with the chosen primer pair, did not lend itself to enzymatic digestion and therefore was subjected to DNA sequencing. The sequence obtained demonstrated the complete homology to previously published sequence of mouse *Muc10*, GENBANK accession number NM_008644.

Muc6 was absent in the middle ear both in vivo and in vitro. Ability of the primer pair to amplify *Muc6* was confirmed using mouse small intestine cDNA in which *Muc6* was

reported to be highly expressed. The obtained small intestine cDNA sequence demonstrated homology to the previously published mouse *Muc6*, GENBANK accession number NM_181729 (data not shown).

The result of mucin gene expression analysis in mouse middle ear epithelium both in vivo and in vitro specimens was summarized in Table 2.

Discussion

Mouse models as an investigative paradigm for pathogenesis in many areas have become increasingly important with sequencing of the mouse genome. This scientific achievement has allowed for development of sophisticated molecular tools to assess genetic function, molecular interactions, protein properties and the ability to create specific *in vivo* constructs through utilization of knock-out, knock-in and knock-down techniques. Given these advantages, utilization of the mouse model in the study of the middle ear (ME) and otitis media (OM) pathogenesis has also received increased attention in recent years with many investigators utilizing mouse models as important research tools [7]. Advances in cell culture techniques have also enabled investigations of middle ear epithelium in culture providing a means to conduct *in vitro* investigations that are not limited by the variables and difficulties inherent in *in vivo* studies. Establishment of primary cell cultures, although technically feasible, is limited by the need for repeated harvest of tissue and inherent variability in underlying cultures obtained. Immortalization of middle ear tissue to allow for consistent and reliable cells for culture has developed as an alternative to repeated primary cell culture [6,9]. Although the transformation of normal epithelial cells into immortalized cells provides challenges with respect to the potential disruption in normal phenotypic or genotypic expression, these techniques have proven effective in multiple laboratories. Importantly, these investigations have also demonstrated genetic, functional and mechanistic similarities between immortalized and normal *in vivo* epithelium rather than differences. [4,10].

Having a reliable *in vitro* method to study MEE provides advantages in conducting focused molecular research into the physiology and pathophysiology of the ME. The results in this manuscript demonstrate complete homology between *in vivo* specimens and the *in vitro* cell culture model of mouse middle ear epithelium with regards to the mouse mucin genes *Muc1, Muc2, Muc4, Muc5AC* and *Muc5B* which have previously been demonstrated to be important in otitis media and otitis media with effusion [11]. In addition, this study characterized the expression of a number of new mucin genes that have been identified in other tissues and in human MEE [4] but have either not previously been reported or compared in these two model systems in the mouse. Of these recently identified mucin genes, *Muc9, Muc13, Muc15, Muc16, Muc18, Muc19* and *Muc20* were consistently identified in the *in vitro* model of MMEEC as well as in the ME of healthy mice without ME disease. In addition, the two models demonstrated complete homology with respect to expression of these mucin genes. The mucin gene *Muc3*, although less recently identified, was also found to be consistently present in both ME models. Further, in cases where mucin genes were found to not be expressed *in vitro*: *Muc6, Muc7, Muc8, Muc11/12* and *Muc17*; these genes were also found to be absent *in vivo*. However, the mouse genome would suggest that, in the mouse, there do not exist corresponding genes for *Muc 7, Muc 8, Muc 11/12* and *Muc 17*. [12,13] These findings of complete homology of mucin gene expression and non-expression between *in vitro* and *in vivo* tissues would suggest that MMEEC models represent a reasonable surrogate for *in vivo* studies when investigating mouse ME mucin mechanisms.

However, the investigators acknowledge that certain limitations in these results should be considered with respect to glandular architecture and absence of complete cell-cell interactions, such as immunocytes, when considering ME pathophysiology and mucin interactions in this *in vitro* model system.

The importance of mucins in respiratory epithelium, in the ME in particular, and in the pathophysiology of OM has prompted numerous investigations into the function, regulation, and potential modulation of these large (500–600kDa) glycoproteins. Our laboratory and others have detailed the importance of mucin proteins with gel-forming qualities, MUC2 [3], MUC5AC [14–15], MUC5B [16–18] , and the more recently described MUC19 [19], given that these proteins are secreted. These secretory properties allow these mucins to function at the apical surface of the epithelium and beyond as they interact with pathogens and protect the underlying epithelium from invasion and mechanical damage. However, the ability to respond in this fashion also provides the opportunity for gel-forming mucin "over-production" leading to more highly viscous fluids with difficulty in ME clearance and subsequent hearing loss. This current investigation demonstrates that the MMEEC model is analogous to *in vivo* mouse models, as well as published human models, with respect to expression of these important gel-forming mucins (Table 2.) and provides a reasonable platform for further investigations.

It has also been recently recognized that in the human ME a much broader range of mucin genes are expressed than previously thought [4]. Given that individual mucin products have specific characteristics, size and interactions with the underlying epithelium and surrounding environment, including pathogens, it would be anticipated that the other mucin genes expressed also have an important role in the MEE. As such, our laboratory has investigated the ME for each of previously described mucins to assess the potential for expression and, ultimately, functionality of these other mucins in the ME. The work from this current investigation would suggest that there is significant uniformity between the mouse and human in not only the gel-forming mucins but also in other, membrane bound, mucins. Both human and mouse epithelia express MUC1, MUC4, MUC9, MUC10, MUC13, MUC15, MUC16, MUC18 and MUC20. And both epithelia do not express MUC6 or MUC17. However, there are differences in mucin gene expression between the mouse and the human. The lack of expression of *Muc7, Muc8, Muc11/12 and Muc17* is notable in considering potential functions of these glycoproteins. Differential expression of these mucins has been reported in malignant and inflammatory diseases of epithelial cells [20–23].

Differences between human models and mouse models are certainly not unique to the ME. However, future investigations should be cognizant of these variations in designing and interpreting results, at least as they specifically relate to mucin function in the ME. However, the concordance between the MMEEC and mouse *in vivo* tissue samples regarding these genes also suggests that findings in either model system, as they relate to mouse ME physiology, may be translatable to the other mouse model.

Conclusion

This current investigation is the first to comprehensively analyze mouse MEE mucin gene expression. The results demonstrate that the *in vitro* MMEEC model has complete correlation to *in vivo* mouse tissue. There also exists complete homology between these mouse models and previously describe human models for most mucins which have been investigated to date. However, there do exist difference between the mouse and human models which warrant future examination.

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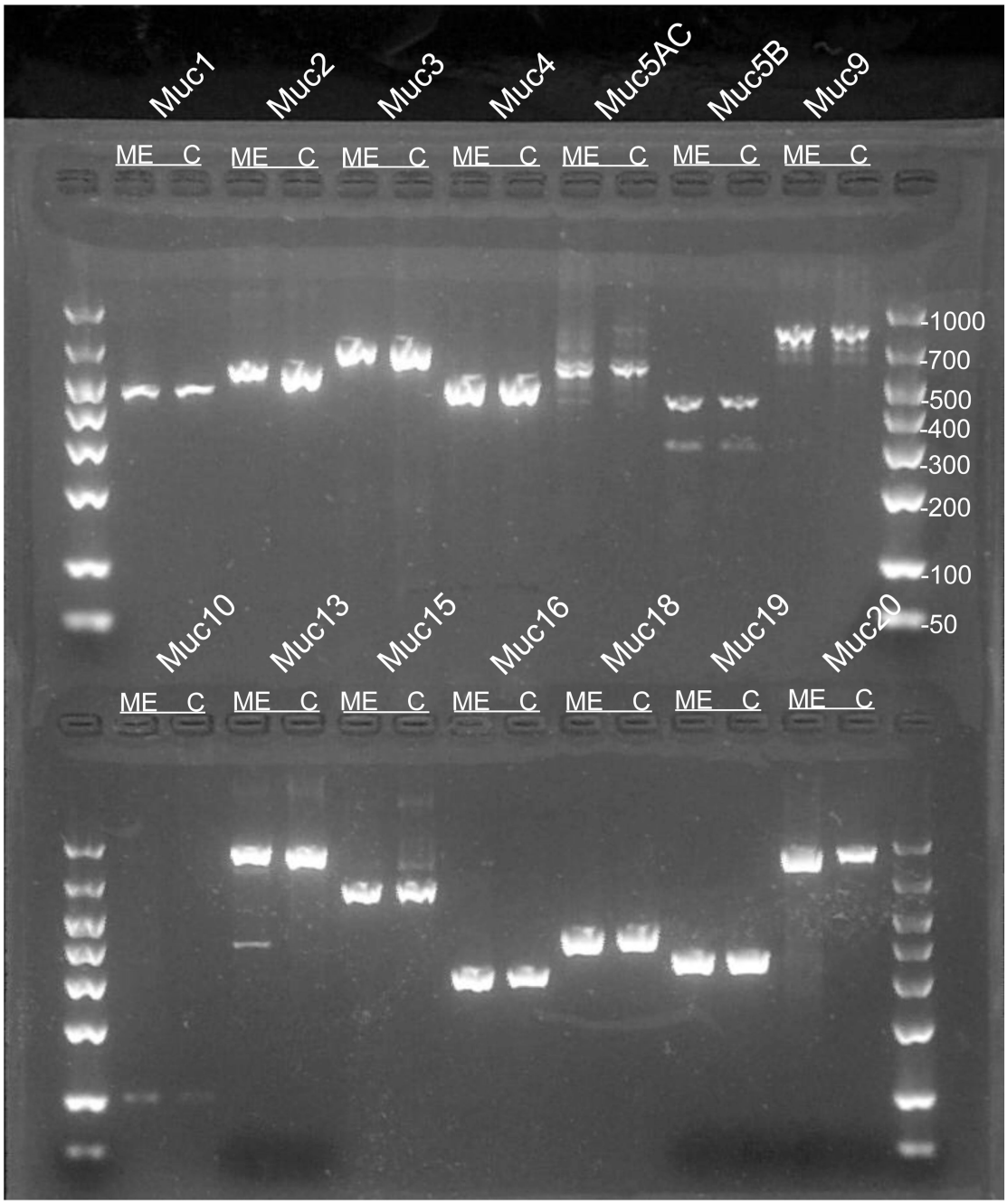

Figure 1. Mucin gene expression in mouse middle ear epithelium

Mucin amplicons generated from mouse middle ear epithelium (ME) and MMEEC (C) were analysis in agarose gel electrophoresis.

Table 1

Mouse mucin primer sequences.

Gene	Fwd Primer	Amplicon (bp)	RE	Expected size (bp)	PrimerReferences
Muc1	TCTCC AGCCA CCAGC CCTCT AA TGGCC ATGGT AGGAG AAACA GG	436	KpnI SphI	259+177 237+199	PMID: 16158528
Muc2	GGGAG GGTGG AAGTG GCATT GT TGCTG GGGTT TTTGT GAATC TC	619	SmaI PstI	402+217 488+131	PMID: 16158528
Muc3	AACTG CAGCT ACGGC AAATG TC AGGTT TCGCC TACCA TCGTA AC	656	EcoRI	529+127	BC058768
Muc4	CATAT TCAAT ACCAC CGGTG TTC AAGGA TGGAA TTGGT GCTTT GTC	466	HinfI	349+117	NM_080457
Muc5AC	CACCA TCTCT ACAAC CCAAA CT TGAGG TCCAG GTCTT TGTGT CT	518	PstI	274+244	PMID: 16158528
Muc5B	GCCCT CACTG CCTCT GCTCC AC TTTTA CAGTG CCAGG GTTTA TT	387	BsmFI DdeI	266+121 248+114+25	PMID: 16158528
Muc6	AGTCC TGCAG CCAGT CGTCA G GCACG CAGGC CTCAT AGTAG	953	BamHI PstI	826+127 674+153+116+10	PMID: 12676567
Muc9	ACTTA TTATG GGTTT CCCCA CC TGGTG GTCTT AGAGA TCCCA GT	745	SmaI	516+229	Riboprobe ID: RP_050505_03_D08
Muc10	GGTTT CATTC CAAGC TCTCC TTAGG AGAAC GGCGA CTGAT	101			PMID: 16514118
Muc13	TCCCT GGGGA CATTA GCA GGCTA GGGAG GGTTC CAA	919	PvuII	617+302	Riboprobe ID: RP_050125_02_A11
Muc15	ATCCT TTACA GGTCT CCGAA CA CTGCA GCCAT CTTTC TCCTA AT	639	DdeI	361+278	Riboprobe ID:RP_060606_02_G12
Muc16	CCAAT CTACT GTACG GAGAA CATG CATAG AGACT GTCCT GATCC AG	315	PstI	183+132	XM_911929
Muc18	GGAAC CAACT ATTCA AGCCA ATG GGTTG AGGGT TGCCA TCTGT C	429	PstI	337+92	NM_023061
Muc19	GATTA TGCGA TTGGT TCATC CT GTGCA ATGTC CCTGA ACTCA TA	349	EcoRI PstI	298+51 176+173	PMID: 12882755
Muc20	ACCCT TTGTA CCGAT GACAG CTCTG AAGAG CAAGC AGTGG ATGCA GATGT TGTAG GATG	884	HindIII	508+376	PMID: 14565953

Table 2

Summary of mucin gene expression in study models: mouse middle ear epithelium; mME, mouse middle ear epithelial culture; MMEEC, human middle ear epithelium; HsME, and human middle ear epithelial culture; HMEEC. (presence; +, absence; −, not tested; nt).

Mucin	mME	MMEEC	HsME	HMEEC
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5AC	+	+	+	+
5B	+	+	+	+
6	−	−	−	−
7	n/t	n/t	+	+
8	n/t	n/t	+	+
9	+	+	+	+
10	+	+	n/t	n/t
11/12	n/t	n/t	+	+
13	+	+	+	+
15	+	+	+	+
16	+	+	+	+
17	n/t	n/t	−	−
18	+	+	+	+
19	+	+	+	+
20	+	+	+	+