In Vitro Effects of Acid and Pepsin on Mouse Middle Ear Epithelial Cell Viability and MUC5B Gene Expression

Bradley B. Block, MD; Elaine Kuo, DVM, MS; Habib G. Zalzal, BA; Hugo Escobar, MD; Mary Rose, PhD; Diego Preciado, MD, PhD

Objective: To examine whether in vitro exposure of mouse middle ear epithelial cells (mMEECs) to conditions that mimic physiologic reflux upregulates Muc5b gene expression and alters cell viability.

Design: In vitro mMEEC model.

Setting: Center for Genetic Medicine Research, Children’s National Medical Center, Washington, DC.

Participants: Cells from the immortalized mMEEC line.

Main Outcome Measures: Cell viability, the quantity of Muc5b messenger RNA abundance, and Muc5b promoter activity.

Results: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays demonstrated an acidic dose-dependent decrease in cell survival, with pH less than 4 significantly decreasing viability at 1 hour. Pepsin had a mild protective effect up to 8 hours, with greater cell viability, in the pH range of 5.0 to 7.6. Reverse-transcriptase polymerase chain reaction demonstrated induction of Muc5b messenger RNA levels over controls after exposure to acidic pH levels of 5.7, and 4 with and without pepsin. Similarly, a pH of 4.0 significantly increased Muc5b promoter activation 5.4-fold. Pepsin at neutral or acidic pH values did not significantly alter Muc5b expression or promoter activity.

Conclusions: Despite decreasing cell viability, acidic pH drives middle ear epithelial Muc5b gene expression in vitro, which perhaps explains how laryngopharyngeal reflux can contribute to otitis media. Pepsin at neutral or acidic pH levels had minimal effects on Muc5b gene expression; thus, although pepsin may be a useful marker for detecting the presence of reflux, our results suggest that acid itself is a more likely pathologic component of gastric juice in the middle ear.


Otitis media (OM) is the most commonly diagnosed pediatric illness and indication for antibiotics in children in the United States. It is the most common cause of pediatric hearing loss. There are multiple pathophysiologic mechanisms that may contribute to the development of OM with effusion, such as eustachian tube dysfunction and obstruction, bacterial infection and biofilms, respiratory tract viruses, adenoid disease, allergy, and, most recently, laryngopharyngeal reflux. Pepsin is a proteinase stored as inactive pepsinogen in the chief cells of gastric mucosa and activated by a pH less than 4.5. In a seminal study, Tasker et al used an enzyme-linked immunosorbent assay with an antipepsin antibody to identify pepsin in middle ear aspirates of children with OM with effusion. They found 59 of 65 effusions to be pepsin positive at concentrations that were 1000 times higher than that of pepsinogen in serum. Since then, these findings have been duplicated multiple times, using different antibodies and enzymatic assays. In the largest study to date, O’Reilly et al confirmed the presence of pepsin in the middle ear of children with recurrent acute OM and OM with effusion, with 20% of patients having pepsin-positive status, in contrast to 1.5% of control individuals.

Despite these mostly co-relational findings, causal mechanisms by which reflux may contribute to the development of middle ear effusion have yet to be elucidated. Diseased middle ears frequently show evidence of mucosal metaplasia with increased concentration of goblet cells. The viscosity of mucus is regulated by mucus, a family of glycoproteins whose protein backbones are characterized by nu-
merous tandem repeats that contain proline and are high in serine and/or threonine residues, the sites of O-glycosylation. They can be classified as either membrane bound, in which they are anchored directly to the bilayer of the cell membrane, or secretory, in which they lack a transmembrane domain and are synthesized as monomers and posttranscriptionally polymerized to form dimers or trimers that comprise a protective mucus layer.\textsuperscript{0,11} The predominant mucin glycoprotein upregulated in OM appears to be MUC5B, a relatively large, high-viscosity secretory mucin.\textsuperscript{12-14} Although mucins in the respiratory tract function primarily to help maintain mucociliary clearance, interact with pathogens, and protect the epithelium, within the confines of the middle ear\textsuperscript{9,11} their overproduction may result in mucous obstruction or stagnation, thus contributing to OM disease.\textsuperscript{15,16} In the esophagus, the presence of stomach acids and bile salts is known to influence the rate of mucin gene expression.\textsuperscript{17,18} Similarly, expression of another secretory airway mucin, MUC5AC, is reported to be induced by acidic pH in laryngeal airway epithelium.\textsuperscript{19} Further, gastroesophageal reflux has been shown to impair normal mucociliary function in nasal and upper airway epithelium.\textsuperscript{20} Finally, animal models that simulate gastric juice upper airway exposure have demonstrated that reflux impairs mucociliary function and increases mucus production.\textsuperscript{21,22} We therefore hypothesized that a mechanism by which laryngopharyngeal reflux may contribute to OM is the upregulation of the genetic expression of the predominant OM mucin, MUC5B, in middle ear epithelium.

This hypothesis was tested in an in vitro mouse middle ear epithelial cell (mMEEC) model by exposing cells to media with acidic pH and subsequently measuring cell abundance, and viability, the quantity of Muc5b messenger RNA (mRNA) expression of the predominant OM mucin, MUC5B, in middle ear epithelium.

Immortalized mMEEC lines\textsuperscript{23,24} were provided by Jizhen Lin, MD (University of Minnesota, Minneapolis). The cells are immortalized by temperature-sensitive simian virus 40 and are thus programmed to proliferate at 33°C and differentiate at 37°C.\textsuperscript{24} The cells were grown in full growth media (FGM) and maintained as previously described.\textsuperscript{24} Before experimentation, cells were transferred to a 37°C, 5% carbon dioxide humidified atmosphere in serum-free media. Cells were then grown on plastic or on permeable supports (Transwells; Corning Inc., Corning, New York) submerged in FGM until they achieved confluence. For some experiments, cells were grown at an air-liquid interface (ALI) for 14 days in serum-free media. Stimulants were applied to the basal side of the cells. These ALI conditions have been reported to further differentiate middle ear cells, with diffuse formation of goblet cells, mimicking in vivo OM metaplastic epithelium.\textsuperscript{25}

For experimental stimulation, acidic pH was established by adjustment of serum-free FGM with 1M sodium hydroxide and/or hydrochloride to the appropriate pH before sterile filtering the media through a 40-µm filter. For some experiments, porcine pepsin (Sigma-Aldrich Corporation, St Louis, Missouri) was then added to the media at a final dose of 2 mg/mL, and the pH was retitrated to ensure no measurable difference in the set pH level.

**MTT COLORIMETRIC ASSAY**

Before testing for pH-induced mucin production, the lowest pH at which in vitro mMEECs remain metabolically active had to be established. This has been previously reported in esophageal epithelia\textsuperscript{26} but not middle ear epithelia. Cells were passaged onto 96-well plates and grown at 33°C in a 5% carbon dioxide humidified atmosphere for 48 hours before the medium was changed to serum-free media at 37°C. The mMEECs were exposed to a pH of 3.0 to 5.5 at intervals of pH 0.5, with a control of 7.6 (the pH of serum-free media), for 1, 2, 4, and 8 hours, with and without 2 mg/mL of porcine pepsin (Sigma-Aldrich Corporation). 6 wells per condition. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich) was then performed.

**REAL-TIME REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION**

Before acid exposure, mMEECs were grown at an ALI for 14 days to allow for differentiation.\textsuperscript{24} During this time, media were changed every 2 to 3 days. Once differentiated, the cells were exposed to pHs of 4.0, 5.7, and 7.6 for 30 minutes. The acidic media was then removed, and cells were kept in serum-free media for 2 and 8 hours before harvesting RNA by means of TRIzol (Life Technologies Corporation, Carlsbad, California). This short bolus of acidic exposure has been used previously to study epithelial mucin gene activation effects.\textsuperscript{19} RNA was precipitated with isopropyl alcohol, washed, and dissolved in ribonuclease-free water. Reverse-transcriptase polymerase chain reaction (RT-PCR) was then performed, as described previously.\textsuperscript{26} Generated complementary DNA was then used for real-time RT-PCR, by the use of specific pairs of primers as follows: Muc5b forward primer, 5’-AAGAGGAGGTGTCCAGGT-3’ and Muc5b reverse primer, 5’-ACATGGCAGGGTTATGC-3’ (GenBank accession No. AJ311906). As an internal control, β-actin was used, and primers for mouse β-actin were obtained from Gene Link (Hawthorne, New York). The 307 and 349 base pairs (bp) are Muc5b and β-actin PCR amplicons, respectively. Real-time RT-PCR was performed on the generated complementary DNA products in a sequence detection system (ABI Prism 7700; Applied Biosystems Inc, Foster City, California) by means of a master mix (Power SYBR Green PCR Master Mix; Applied Biosystems Inc). As an internal control for normalizing Muc5b mRNA levels in control and experimental samples, β-Actin was used. Relative quantification of Muc5b mRNA in control and experimental samples was obtained by means of the standard curve method.
CONSTRUCTION OF MURINE Muc5b LUCIFERASE REPORTER PLASMID

The promoter sequence for Muc5b was identified on GenBank as accession No. AY744445. The following primers tagged with 5’ restriction enzyme sequences were obtained from Integrated DNA Technologies (Coralville, Iowa): F – 1785 5’GACTCTCGAGGGATCCTCAAGGCCCAC T 3’ and R + 47 5’GACTAAGGCTCTGGGGCAGTACAGA3’ (XhoI restriction sites on the F primers’ 5’ end and HindIII restriction site on the R primer 5’ end). We performed PCR using these primers and mouse liver genomic DNA as the template. After purification, resulting products were restricted and ligated to the PGL3 vector (Promega, Madison, Wisconsin). After confirmation of the nucleotide sequences, the −1785-bp nucleotide Muc5bLuc promoter plasmid (−1785Muc5bLuc) was then transformed in competent DH5α cells and amplified (MaxiPrep kits; Promega).

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

−1785Muc5bLuc or PGL3 (empty vector) reporter plasmids were transiently transfected into mMEECs for luciferase assays as follows. Cells were passaged onto plastic 12-well plates (Fisher Scientific, Pittsburgh, Pennsylvania) in FGM and grown at 33°C in a 5% carbon dioxide humidified atmosphere. Once 80% confluent, they were transferred to a 37°C, 5% carbon dioxide humidified atmosphere in serum-free media. Cells were cotransfected with the luciferase plasmids (1 µg/mL) and a pCMV-βGal reporter construct (0.1 µg/mL) (Clontech Laboratories Inc, Mountainview, California) in Opti-MEM medium containing 3 µg/mL of Lipofectamine 2000 (Life Technologies Corporation). After 16 hours, the medium was removed and the cells were stimulated for 30 minutes with serum-free media that had been adjusted to acidic pH. The pH values of 7.6, 6.0, 5.0, and 4.0 were used, with and without 2 mg/mL of porcine pepsin. The media were then switched to serum-free media for 7.5 hours. Cell lysates were then procured by scraping the cells in lysis buffer (Applied Biosystems Inc) supplemented with 0.5mM dithiothreitol. The relative luciferase activity was determined with the dual light reporter gene assay kit (Tropix Inc, Medford, Massachusetts) and a Monolight 2010 plate luminometer (Analytical Luminescence Laboratory, San Diego, California) according to the instructions of the manufacturers. Results for relative luciferase units were determined as a count ratio of the −1785Muc5bLuc construct over the pCMV-βGal reporter to normalize for transfection efficiency and subsequently normalized by background empty vector PGL3/pCMV-βGal counts for each experimental condition.

STATISTICAL ANALYSIS

The statistical difference between the experimental and control groups for all experiments was determined by 2-tailed t tests. Significance level was set at P < .05.
Stimulation of ALI-differentiated mMEECs with pH 4.0 and 5.5 did not have a statistically significant effect on Muc5b expression after 2 hours, with only 1.2- and 1.8-fold increases over neutral pH, respectively (data not shown). After 8 hours, however, a statistically significant dose-response acid effect was seen on Muc5b mRNA production, with a 1.9-fold and 5.8-fold increase over neutral pH control with pH values of 5.7 and 4, respectively (Figure 2A). Addition of 2 mg/mL of porcine pepsin mitigated the effect at pH 4 but enhanced the effect at pH 5.7 (P < .05 compared with neutral pH). Bar graphs without pepsin are shaded. Error bars indicate standard error.

**EFFECT OF ACIDIC pH ON ACTIVITY OF THE MURINE Muc5b PROMOTER IN mMEECs**

To determine whether the observed increases in Muc5b mRNA levels with acidic pH were owing to increased transcriptional regulation, studies were performed to determine whether acidic pH increased promoter activity of a –1785-bp luciferase reporter construct. Luciferase assays (Figure 3) demonstrated statistically significant 1.7-fold, 1.9-fold, and 5.41-fold relative luciferase unit activation of the –1785Muc5bLUC promoter construct in mMEEC with pH values of 6 (P < .001), 5 (P = .01), and 4 (P = .002), respectively, compared with neutral pH without pepsin. Similar to previous data, the addition of pepsin somewhat mitigated the effects at pH 4 (which reduced the fold activation to 3.8), but increased promoter effects were still noted. The results suggest that the observed increase in Muc5b mRNA expression induced by acidic pH (Figure 2A) is at least partially regulated at the transcriptional level.

**COMMENT**

Pepsin has been found in the middle ear of pediatric patients with OM with effusion and recurrent acute OM at levels higher than those of control individuals (patients with healthy middle ears undergoing cochlear implant surgery and having saline tympanic lavage). Because this result has only recently been demonstrated, the mechanisms by which reflux contributes to middle ear disease have yet to be elucidated. Muc5b is preferentially upregulated in OM with effusion and thus can be assayed for in vitro to infer whether pathologically relevant stimulants contribute to OM.

Real-time RT-PCR data showed a statistically significant increased acidic dose stimulation effect on Muc5b transcription at 8 hours after a short bolus of acid. This finding suggests that a mechanism by which reflux contributes to middle ear effusions is by an effect on gene regulation. The refluxate induces the transcriptional up-regulation of the highly viscous mucin Muc5b, perhaps thus ultimately leading to “glue ear.” Moreover, luciferase data indicate that gastric reflux indeed may signal intracellularly to directly enhance mucin gene promoter activity. This is the first report, to our knowledge, of either of these findings in middle ear cell systems. Our results are in line with those of Samuels et al., which showed that a similar course of exposure induced the transcription of Muc5ac in laryngeal airway epithelium at pH 2 and that the presence of pepsin abated this effect. Ongoing experiments in our laboratory are being performed to determine whether the increased expression of Muc5b mRNA translates into increased production of Muc5b mucin in mMEEC secretions.

Pepsin has proteolytic activity with a maximum active range at pH 2.3 to 4.5. Some collagenase activity re-
maintained at pH 5.5, but at pH 7.0 pepsin begins to denature. Importantly, at pH 5.7 pepsin showed enhancement of the acid effect on Muc5b mRNA levels at 8 hours (Figure 2). Reasons for this result are unclear, but a pH of 5.7 is more relevant to what has been found in the middle ear, where the pH levels of effusions have been reported to range from 6.0 to 7.6. Therefore, pepsin should have minimal, if any, activity in the middle ear and should be starting to denature. If pepsin plays a pathologic role in OM, it must be as transient as the reflux episode itself.

The pepsin used in this study was porcine, whereas the cells were murine. However, pepsin has evolved to have proteolytic activity against consumed matter, not necessarily an animal’s own epithelial cell layer, and comparative biochemical studies have established that there are striking structural similarities and conservation across species. Despite this, there are also phylogenetic functional protein differences. This finding is a limitation of our study. The dose of pepsin used in our study, 2 mg/mL, is high. This pepsin dose was used because, at a concentration of 2 mg/mL, pepsin in solution has the same enzymatic activity as pepsin from crude gastric juice. Patients with symptoms of Barrett esophagus while supine were found to have a pepsin concentration of 300 μg/mL in their esophagus. Thus, we selected this dose because the enzymatic activity of pepsin remains physiologic, and the higher dose theoretically should increase the likelihood of noticing an effect.

Interestingly, our results indeed showed that pepsin conferred improved cell viability to acidic pH exposure. How this result exactly translates to the clinical condition of acid reflux into the middle ear is unclear, but the findings suggest that perhaps pepsin may have a protective effect through digestion of other proteins, either in the cells themselves or in the media in vitro, or by affecting proteins important in the inflammatory response in vivo. Another possibility is that the mitigating effect may be unrelated to the normal biological activity of pepsin, as previously mentioned. Future experimenters could consider using an inactive form of pepsin as a control or adding pepsinogen as the precursor. As demonstrated by Jiang et al in esophageal epithelium, cell viability after exposure to acid was found to be directly proportional to the pH, which implies that the acid itself is the pathologic agent in refluxate. Along the same lines, addition of pepsin did not significantly alter the acid effects on Muc5b transcription or promoter activity. Thus, although pepsin may not be the pathologic agent in reflux, it may be a useful marker for detecting the presence of refluxate in the middle ear.

Notably, the effects of pH 4 on Muc5b transcription as measured by RT-PCR were mitigated by the addition of pepsin, which instead enhanced the effects of pH 5.7 on Muc5b transcription. The reasons for this are unclear, but it is possible that dissolved pepsin in solution is somehow chelating or neutralizing the acid, effectively reducing its potency or protecting the cells from noxious acidic intracellular effects. However, the addition of pepsin to the media did not measurably alter the adjusted pH levels of the media. Finally, a limitation of our study is that the luciferase assays were performed on mMEECs grown on plastic, whereas RT-PCR measures of gene transcription were performed on differentiated cells grown in an ALI. Admittedly, in our hands it has proven difficult to transiently transfect the mMEECs after 14 days of ALI-differentiating conditions. It appears that the transfection process is either toxic to the differentiated cells or gene transfer is not efficient in these. At the same time though, we have found that measures of Muc5b mRNA production are clearly more robust when the cells are differentiated. This makes sense because in ALI there are more goblet cells present, which ultimately are the cells responsible for producing mucus.

In conclusion, the relationship of acid reflux to OM pathogenesis remains unclear. Causality has never been demonstrated for either acidic pH or pepsin. Our current in vitro data suggest that only reflux agents may play a role in the increase of mucus abundance in the middle ear. In summary, we show that acidic pH reduces mMEEC viability but induces Muc5b transcription and promoter activation in vitro, which perhaps explains how laryngopharyngeal reflux can contribute to OM. Furthermore, pepsin itself had minimal effects on Muc5b gene expression, which indicates perhaps that acid is a more likely pathologic component of gastric juice in the middle ear.

Submitted for Publication: April 2, 2009; final revision received July 7, 2009; accepted August 3, 2009.

Correspondence: Diego Preciado, MD, PhD, Division of Pediatric Otolaryngology–Head and Neck Surgery, Children’s National Medical Center, 111 Michigan Ave NW, Washington, DC 20010 (dpreciad@cmmc.org).

Author Contributions: Drs Block and Preciado had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Block, Kuo, Escobar, Rose, and Preciado. Acquisition of data: Block, Kuo, Zalzal, and Preciado. Analysis and interpretation of data: Block, Kuo, Rose, and Preciado. Drafting of the manuscript: Block, Kuo, and Preciado. Critical revision of the manuscript for important intellectual content: Block, Kuo, Zalzal, Escobar, Rose, and Preciado. Statistical analysis: Kuo and Preciado. Obtained funding: Rose and Preciado. Administrative, technical, and material support: Block, Kuo, Zalzal, Escobar, Rose, and Preciado. Study supervision: Rose and Preciado.

Financial Disclosure: None reported.

Funding/Support: This work was supported by a Triologic Society Career Development Award and by an Avery Scholar Research Award from Children’s National Medical Center (Dr Preciado).

Previous Presentation: This study was presented at the American Society for Pediatric Otolaryngology Annual Meeting; May 25, 2009; Seattle, Washington.

REFERENCES

