Characterization of mucins in human middle ear and Eustachian tube

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Received 25 April 2000; accepted in final form 3 January 2001

Mucins are important glycoproteins in the mucociliary transport system of the middle ear and Eustachian tube. Little is known about mucin expression within this system under physiological and pathological conditions. This study demonstrated the expression of MUC5B, MUC5AC, MUC4, and MUC1 in the human Eustachian tube, whereas only MUC5B mucin expression was demonstrated in noninflamed middle ears. MUC5B and MUC4 mucin genes were upregulated 4.2- and 6-fold, respectively, in middle ears with chronic otitis media (COM) or mucoid otitis media (MOM). This upregulation of mucin genes was accompanied by an increase of MUC5B- and MUC4-producing cells in the middle ear mucosa. Electron microscopy of the secretions from COM and MOM showed the presence of chainlike polymeric mucin. These data indicate that the epithelium of the middle ear and Eustachian tube expresses distinct mucin profiles and that MUC5B and MUC4 mucins are highly produced and secreted in the diseased middle ear. These mucins may form thick mucous effusion in the middle ear cavity and compromise the function of the middle ear.

Mucins are a family of glycoproteins with distinct biological structure and physiological functions. Mucins are involved in host cellular protection, proliferation, host-pathogen adhesion, and tumor biology. There are two classes of mucins, membrane-bound and secretory, which differ in biological structure and cellular location. Mucins with a transmembrane domain are designated as membrane-bound mucins. Membrane-bound mucins anchor directly to the bilayer of a membrane and play a role in cellular shielding, adherence, and cellular proliferation of the mucosal epithelium. Secretory mucins lacking a transmembrane domain are synthesized in a dimeric or trimeric manner, are packed into mucous granules, and are secreted on an apical surface to form a mucus layer that protects the epithelium and defends against invading microorganisms. Membrane-bound mucins may also be produced as a secreted or soluble form in certain tissues. Detailed information on mucins and mucin genes has appeared in several review articles (16, 26, 34, 35).

To date, at least nine distinct mucins from the human body have been identified and designated as MUC1–4, MUC5AC, MUC5B, or MUC6–8 (16, 26, 35, 39), depending on the date at which they were first described. MUC1, MUC3, and MUC4 are considered to be membrane-bound mucins; MUC2, MUC5AC, MUC5B, MUC6, and MUC7 are thought to be secretory mucins; MUC8 is still unclassified.

Genes for secretory mucins MUC2, MUC5AC, MUC5B, and MUC6 are known to be clustered at the locus on chromosome 11p15.5 (3, 14, 18, 42) and are believed to be derived from the same ancestral gene, given their genetic organization and structural similarity. MUC1 is located on 1q21–24 (15, 41), MUC3 on 7q22 (19), MUC4 on 3q29 (31), MUC7 on 4q13–21 (7), and MUC8 on 12q24.3 (39).

In the middle ear, mucins are important structural components of the mucociliary transport system that covers cellular surfaces, provides transport toward the nasopharyngeal orifice, and cleans the middle ear cavity. Because middle ear mucins are able to bind to proteins in the outer membrane of bacteria (32), they are thought to play an essential role in evacuating middle ear pathogens that ascend along the Eustachian tube. Under disease conditions in the middle ear, however, alterations in mucin metabolism are thought to contribute to dysfunction of the mucociliary transport system (36, 37).

The profile of mucin genes that are expressed in the normal human Eustachian tube and middle ear is unknown at the present time, as are possible alterations that may occur in mucin gene(s) under disease
MUCIN GENES IN MIDDLE EAR AND EUSTACHIAN TUBE

conditions. Studies of mucin gene expression in these two structures are hindered by difficulties in obtaining normal human tissues and are limited by the extremely small amounts of middle ear effusion available for study. Understanding mucin metabolism under normal physiological conditions and the alterations that occur during disease states is key to understanding the molecular mechanisms of middle ear diseases. This study was designed to characterize mucin gene expression in the normal middle ear and Eustachian tube and in the middle ear of humans with otitis media, which is the most common cause of acquired hearing loss in adults and impaired language development and communication disorders in children.

MATERIALS AND METHODS

Procurement of human middle ear and Eustachian tube tissues and middle ear effusions. Eight middle ear and Eustachian tube tissue specimens were obtained at immediate autopsy (3–6 h after death) of the temporal bones of four male patients, ages 50–70 yr, who died from heptocellular carcinoma, pneumonia, coronary artery disease, and infarcted bowel, respectively. None of the patients had a history of middle ear disease. Middle ear specimens were dissected from the inferior area of the promontory, and Eustachian tube specimens were dissected from the orifice of the nasopharynx to the orifice of the tympanum. Twenty middle ear tissue specimens from the same areas described above were biopsied from the middle ears of 20 patients diagnosed with otitis media [chronic otitis media (COM) or mucoid otitis media (MOM)]. A total of 3 ml of middle ear effusion collected at surgery from six of the patients with mucoid otitis media (MOM) were pooled for mucin analyses. Very little or no middle ear effusion was evident in 14 patients as a result of perforation of the tympanic membrane.

Middle ear effusion (3 ml) was used for purification of mucins. Identity of the purified middle ear mucins was determined by ELISA. Morphometric analysis of the purified middle ear mucin was completed by electron microscopy.

Ten middle ear biopsies (8 COM and 2 MOM), three middle ear autopsy specimens, and three autopsy Eustachian tube autopsy specimens were fixed in 10% formalin overnight, embedded in paraffin, and sectioned at a thickness of 5 μm using the method described previously (27). Surgical tissues of human gallbladder, small intestine, stomach, and submaxillary glands served as positive or negative controls for mucins. Ten middle ear biopsies (8 COM and 2 MOM), five middle ear autopsy specimens, and five Eustachian tube autopsy specimens were used for total RNA isolation using the method described by Chomczynski and Sacchi (10). Surgical specimens of human stomach, colon, submaxillary glands, and fibroblast tissues served as positive or negative controls for mucins in these specimens.

To study the expression of mucin mRNA and proteins, in situ hybridization, Northern blot, or immunohistochromistry was performed as described below. Mucin cDNA probes and antibodies used in this study are listed in Tables 1 and 2. The antibodies MRB and M3P and the cDNA probes SIB139 and SMUC-41 were gifts from Drs. Young S. Kim, James R. Gum, Jr., and Carol Busabaum. The 139H2 antibody and cDNA probe pum 24p were donated by Drs. John Hilkens and Dallas M. Swallow, respectively.

Analyses of mucin mRNA by in situ hybridization and Northern blot. For in situ hybridization, linearized vectors with cDNA probes for MUC1, MUC2, MUC3, MUC5AC, MUC5B, and MUC6 tandem repeat units were transcribed into sense or antisense riboprobes with isotope labeling with 35S in vitro by T7/T3/Sp6 phage RNA polymerase, using the MAXscript kit (Ambion, Austin, TX). MUC4, MUC7, and MUC8 oligo cDNA probes were linked with T7/T3 promoter sequences and then transcribed into sense or anti-sense riboprobes using the same kit. In situ hybridization of middle ear, Eustachian tube, and control tissues was performed as described previously (27). Briefly, middle ear and Eustachian tube sections were deparaffinized, digested with protease K, hybridized with mucin MUC1–8 riboprobes, washed under stringent conditions [5× sodium chloride and sodium citrate (SSC, pH 7.0) and 10 mM dithiothreitol (DTT) at 42°C for 30 min; 2× SSC, 50% formamide, and 10 mM DTT at 60°C for 20 min; and RNA washing solution (0.1 M Tris·HCl at pH 7.5, 0.4 M NaCl, and 50 mM EDTA) for 10 min, two times], exposed to emulsion solution, and counterstained with hematoxylin.

For Northern analysis, the same cDNA probes as for tandem repeat units were used. Vectors with cDNA probes were digested with EcoRI, BamHI, or PstI endonuclease and labeled with 32P using the Prime-a-Gene labeling system (Promega, Madison, WI).

Table 1. Mucin cDNA probes

<table>
<thead>
<tr>
<th>Mucin Gene</th>
<th>Chromosomal Locus</th>
<th>cDNA Clone</th>
<th>Specificity</th>
<th>TR Amino Acid Sequence</th>
<th>Size (no. of AA)</th>
<th>Reference No.</th>
</tr>
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<tbody>
<tr>
<td>MUC1</td>
<td>1q21q24</td>
<td>pum 24p</td>
<td>MUC1 TR</td>
<td>GSTAPAHGVTSAPDTRPAP</td>
<td>20</td>
<td>15, 41</td>
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<td>MUC2</td>
<td>11p15</td>
<td>SMUC41</td>
<td>MUC2 TR</td>
<td>PTTPPTTTTIVTPPTPTGQT†</td>
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<td>18</td>
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<td>MUC3</td>
<td>7q22</td>
<td>SIB 139</td>
<td>MUC3 TR</td>
<td>HSTPSPTSSITTETTS</td>
<td>17</td>
<td>17, 19</td>
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<tr>
<td>MUC4</td>
<td>3q29</td>
<td>M4oligo</td>
<td>MUC4 TR</td>
<td>(T)SS(A)ST(GHA/T)P(L)P(VD)</td>
<td>16</td>
<td>30, 31</td>
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<tr>
<td>MUC5AC</td>
<td>11p15</td>
<td>4F</td>
<td>MUC5AC TR</td>
<td>TTSTTSAP</td>
<td>8</td>
<td>3, 22</td>
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<td>MUC5B</td>
<td>11p15</td>
<td>hGBM4-1</td>
<td>MUC5B TR</td>
<td>STTPTAHTHTHEQTATTTPTATGATAP</td>
<td>28</td>
<td>14, 25</td>
</tr>
<tr>
<td>MUC6</td>
<td>11p15</td>
<td>N/A</td>
<td>MUC6 NTR</td>
<td>Not applicable (N/A)</td>
<td>N/A</td>
<td>30, 31</td>
</tr>
<tr>
<td>MUC7</td>
<td>4q13-21</td>
<td>M7oligo</td>
<td>MUC7 TR</td>
<td>SPSSSSGGTAPSFSQTTTTTTPSPHPPTPHTVIPPPFSTSLVLTPSTGTVIPTPHAQMATSAQH:SSHIHPTPTGPTPPLLAKGTHTAPPP:MTPTSGSQTSSSFTAKTS:SS:SSHSTTHHPEVPTSTPTTTTPNSTGTSFTPVATHTTASRSSLLRPTFTTPHEPPTP</td>
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<tr>
<td>MUC8</td>
<td>12q24.3</td>
<td>M8oligo</td>
<td>MUC8 TR</td>
<td>TTAGPPTSPATTPAPFSAPPSPP</td>
<td>23</td>
<td>7</td>
</tr>
</tbody>
</table>

AA, amino acid; TR, tandem repeat; NTR, nontrant tandem repeat. †Intestinal version of MUC2TR. *Intestinal version of MUC2TR (used in this study).
Table 2. Mucin antibodies

<table>
<thead>
<tr>
<th>Mucin Gene</th>
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<th>Type</th>
<th>Specificity</th>
<th>Reference No.</th>
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<td>PAb</td>
<td>Synthetic MUC2</td>
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</tr>
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<td>PAb</td>
<td>Synthetic MUC3</td>
<td>19</td>
</tr>
<tr>
<td>MUC4</td>
<td>Chicken M4P</td>
<td>PAb</td>
<td>Synthetic MUC4</td>
<td>Ho, unpublished</td>
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<td>PAb</td>
<td>Synthetic MUC5AC TRP</td>
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<tr>
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<td>PAb</td>
<td>Synthetic MUC5B</td>
<td>Ho, unpublished</td>
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<tr>
<td>MUC6</td>
<td>Chicken M6P</td>
<td>PAb</td>
<td>Synthetic MUC6</td>
<td>21</td>
</tr>
</tbody>
</table>

MAb, monoclonal antibody; PAb, polyclonal antibody; TRP, tandem repeat peptide.

Mega, Madison, WI). Northern analysis of middle ear mRNA was performed as described previously (27). Total RNA (5–20 μg) was denatured and size-separated by electrophoresis on a 1.0% agarose gel containing 2.2 M formaldehyde, blotted on a nylon membrane, cross-linked by ultraviolet light, hybridized by 32P-labeled mucin MUC1 cDNA probe at a radioactivity of 2 × 106 counts·min⁻¹·ml⁻¹, and washed under stringent conditions. The blot was stripped with 0.1% SDS and reprobed with MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC8, and β-actin cDNA probes. To make certain that Northern analysis was quantitative, MUC5B and MUC4 non tandem repeat cDNA probes were also used.

Analyses of mucin glycoprotein by immunohistochemistry, ELISA, and electron microscopy. To study the expression of mucins in middle ear and Eustachian tube tissues, specific mucin antibodies (Table 2) were used for identification of their mucin glycoproteins. Immunohistochemistry was performed as described previously (22).

To study the expression of mucins in the middle ear effusion, mucins were purified using the method described previously (28). Briefly, middle ear effusion was suspended in buffer (containing 4 M guanidine hydrochloride, 50 mM Tris·HCl, 5 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride), sonicated for 1 min, homogenized for 1 min, and incubated with 100 mM DTT for 24 h and 250 mM of iodoamylamide for 24 h. Debris and insoluble substances were removed by centrifugation at 8,000 g for 20 min. Effusions were supplemented with cesium chloride at 1.4 g/ml, centrifuged at 1.6 × 10⁶ g for 72 h, and then fractionated at 1 ml/tube. Density, protein, and hexose were determined by bicinchoninic acid assay, and hexose assay for each tube, according to the method described previously (28). Fractions with a density of ~1.4 g/ml and a hexose-to-protein ratio of at least 2:1 were defined as mucins.

Mucins were refined by repeating the cesium chloride gradient centrifugation three times under the same conditions as above, filtered through an AP-100 device (Amicon) to cut off molecules (<100 kDa), and dialyzed against distilled water at 4°C with stirring. To confirm the identity of purified middle ear mucins, ELISA was performed using the method described previously (38). Briefly, 1.5 μg of middle ear mucin and 1 μg of cervix mucin (control) were loaded on 96-well plates for 2 h at room temperature. Plates were blocked with 5% BSA in Tris-buffered saline (TBS: 25 mM Tris, pH 7.4, 140 mM NaCl, and 3 mM KCl) overnight at 4°C. The plates were washed with 0.02% Tween 20 in TBS and incubated with the primary antibodies (chicken anti-human MUC5B and MUC4 in 1:1,000 dilution) for 3 h at room temperature. After washing as before, peroxidase-conjugated rabbit anti-chicken IgY (1:2,000) was added for 1.5 h. Color development was performed with 3,3′,5,5′-tetramethylbenzidine and quenched with 8 M H2SO4. Bound antibody was quantified by measuring the absorbance at 450 nm with a TiterTek spectrophotometer. Preimmune antibodies were used as negative controls.

To determine the molecular image of the purified middle ear mucins, middle ear mucin was applied to thin carbon-coated grids and stained with 1% (wt/vol) uranyl acetate. Micrographs were recorded with a Jeol 1010 electron microscope at 60 kV at a magnification of ×50,000. The printed images were digitalized on a Linotype-Hell flatbed scanner interfaced to a Macintosh computer at a pixel size of ~0.4 μm per pixel level. Adobe Photoshop software was used to apply a high-pass filter to suppress the very low spatial frequencies and thus increase the signal-to-noise ratio of the images.

RESULTS

Mucin gene expression in human Eustachian tubes. The profile and cellular location of mucin gene expression in the Eustachian tube are presented in Fig. 1. Mucin gene antisense riboprobes (MUC1, MUC4, MUC5AC, and MUC5B) hybridized positively with the apical surface of the Eustachian tube epithelium (Fig. 1, M1, b), the whole epithelial layer (Fig. 1, M4, b), epithelium (Fig. 1, M5AC, b and M5B, b), and submucosal glands (Fig. 1, M5B, b), respectively. Expression of MUC4 and MUC1 was not as abundant as that of MUC5B and MUC5AC. No other mucin genes (MUC2, MUC3, MUC6, MUC7, and MUC8) were expressed in the epithelium of the Eustachian tubes. No mucin riboprobes other than MUC5B hybridized with the submucosal glands of the Eustachian tube. MUC-positive control tissues demonstrated hybridizing signals for MUC1 (gallbladder), MUC2 (small intestine), MUC3 (small intestine), MUC5AC and MUC6 (stomach), and MUC5B and MUC7 (submaxillary glands) antisense riboprobes, respectively. Northern analysis of Eustachian tube specimens is presented in Fig. 2. Mucin mRNA transcripts in the Eustachian tube were hybridized with MUC5B, MUC5AC, MUC4, and MUC1 but not with MUC2, MUC3, MUC6, MUC7, or MUC8 cDNA probes. β-Actin housekeeping gene expression for loading controls is shown in Fig. 2, bottom.

Immunohistochemistry demonstrated positive staining of Eustachian tube tissue sections with MUC5B, MUC5AC, MUC4, and MUC1 mucin antibodies (Fig. 3) but not with their preimmune sera. The cellular distribution of the mucins was consistent with that of mucin mRNA. MUC5B mucin glycoprotein was found in the epithelium and submucosal glands of the Eustachian tube (Fig. 3i). MUC5AC mucin glycoprotein was found in the epithelium but not submucosal glands of the Eustachian tube (Fig. 3f). MUC4 mucin glycoprotein was found throughout the epithelium (Fig. 3d). Low levels of MUC1 mucin glycoprotein were found in the surface epithelium. Neither MUC4 nor MUC1 mucin
glycoproteins were found in submucosal glands of the Eustachian tube.

Expression of mucin genes in noninflamed middle ears and upregulation of MUC5B and MUC4 mucin genes in inflamed middle ears. Expression of MUC5B mucin gene was detected in the noninflamed middle ears. In situ hybridization showed spotty positive signals with MUC5B mucin antisense riboprobe (Fig. 4A, M5B, b). Consistent with this, Northern analysis demonstrated only MUC5B mucin gene expression in middle ear epithelium (Fig. 2f). No other mucin gene expression was detected by in situ hybridization or Northern blot (data not shown). Immunohistochemistry detected only very weak expression of MUC5B (Fig. 3j) mucin, supporting the findings of in situ hybridization and Northern analysis.

Fig. 1. Expression of mucin genes in the human Eustachian tube (ET). ET sections were hybridized with mucin antisense riboprobes (second and fourth columns, positive signals) and with mucin sense riboprobes (first and third columns, negative signals). MUC1 (M1), MUC4 (M4), MUC5AC (M5AC), and MUC5B (M5B) mucin genes were expressed in the epithelium of the ET (arrows, M1, b; M4, b; M5AC, b; and M5B, b), whereas MUC2 (M2), MUC3 (M3), MUC6 (M6), MUC7 (M7), or MUC8 (M8) were not (M2, b; M3, b; M6, b; M7, b; and M8, d). Note the distribution of mucin gene expression: MUC5AC mucin mRNA was distributed exclusively in the epithelium (M5AC, b, arrows), and the MUC5B mucin mRNA was found in both epithelium (arrows) and submucosal glands (M5B, b, arrowheads). Positive controls of MUC1 (M1, d, gallbladder epithelium (GB)), MUC2 (M2, d, goblet cells of the small intestine (SI)), MUC3 (M3, d, SI), MUC5AC [M5AC, d, stomach epithelium (SM)], MUC5B (M5B, d, submaxillary mucus glands (SG)), MUC6 (M6, d, SM), and MUC7 (M7, d, SG) all demonstrated hybridizing signals. M1, a and b, represents transverse sections of the ET; all others were parallel sections of the ET. Original magnification was ×400 for M1, M4, M7, and M8 and ×80 for all others.

Fig. 2. Northern analysis of middle ear and ET mucin gene expression. Lane 1, middle ear (M); lane 2, ET (E); lane 3, stomach (S); lane 4, colon (C); lane 5, submaxillary glands (G); lane 6, fibroblast (F, negative control for mucin genes). MUC1 (M1) was weakly expressed in the ET but not in the middle ear. MUC4 (M4) was expressed in the ET but not in the middle ear, as was MUC5AC (M5AC). MUC5B (M5B) was expressed in both the middle ear and ET. None of the MUC2 (M2), MUC3 (M3), MUC6 (M6), MUC7 (M7), or MUC8 (M8) mucin genes was expressed in the middle ear or ET. Note that controls for MUC1 (S or G), MUC2 (C), MUC3 (C), MUC4 (C), MUC5AC (S), MUC5B (G), MUC6 (S), and MUC7 (G) were positive, whereas controls for MUC1 (C), MUC2 (S or G), MUC3 (S or G), MUC4 (S or G), MUC5AC (C and G), MUC5B (S and C), MUC6 (C or G), MUC7 (S or C), and MUC8 (S, C, or G) were negative. The size of MUC1, MUC4, MUC5AC, and MUC5B mucin mRNA transcripts in the ET was ~6 kb, 14–15 kb, 17–18 kb, and 17.5 kb, respectively. Note that MUC4 mucin mRNA transcript in autopsy specimens appeared to be partially degraded. β-Actin gene expression (bottom) was used as a loading control for RNA.
In the inflamed middle ears, expression of not only MUC5B but also MUC4 mucin genes was detected. In situ hybridization demonstrated extensive and strong positive signals with MUC5B mucin antisense riboprobe (Fig. 4A, M5B, d) and extensive positive signals with MUC4 mucin antisense riboprobe (Fig. 4A, M4, d). Semiquantitative Northern analysis (Fig. 4B) with both tandem repeat and nontandem repeat cDNA probes.
probes showed MUC5B and MUC4 mucin gene expression to be ~4.2- and 6-fold, respectively, higher in the inflamed middle ear specimens than in the noninflamed specimens after normalization of expression by the housekeeping gene β-actin (Fig. 4C). No mucin gene expression other than MUC5B and MUC4 was detected by Northern analysis and in situ hybridization (data not shown). Immunohistochemistry demonstrated extensive expression of MUC5B and MUC4 mucins in the entire middle ear epithelium with COM (Fig. 3k, COM), supporting the findings of Northern and in situ hybridization.

An increase of the MUC5B- and MUC4-producing cells was obvious in the inflamed middle ears (Fig. 3, h and k, COM) but not in the noninflamed middle ears (Fig. 3, g and j, control).

Identity and morphology of the middle ear mucins. ELISA and electron microscopy were used to identify the mucins in middle ear secretions and study their morphology. After ultracentrifugation of middle ear effusion in cesium chloride solution, the hexose assay demonstrated two peaks among the 12 fractions of middle ear effusion: one at fraction 7 and another at fraction 10 (Fig. 5A). Both fractions were considered to
be mucins, since they displayed the following characteristic features of a mucin: a high density between 1.35 and 1.45 g/ml and a high hexose-to-protein ratio of ~2:1 or above. Mucins in both fractions were recognized by MUC5B and MUC4 mucin antibodies (Fig. 5B). Fraction 10 was rich in MUC5B, in contrast to fraction 7; therefore, fraction 10 was submitted to further examination by electron microscopy. The middle ear mucins in fraction 10 demonstrated a branched strand containing linear portions and thickened bulb-like portions in the central region. The linear portions of the strands between bulbs varied between 60 and 180 nm in length, with a thickness of 4–5 nm. Globular domains 4–5 nm in diameter were interspersed along the linear portions of the mucin strands (Fig. 6, inset).

A summary of findings on mucin mRNA and glycoprotein of noninflamed Eustachian tube and middle ear specimens from immediate autopsies and inflamed middle ear specimens from biopsies is presented in Table 3.

**DISCUSSION**

The human Eustachian tube, also known as an auditory tube because of its role in hearing, is deeply embedded in the temporal bone and inaccessible in living humans for the study of its gene profiles in physiological and pathological states. Recently, we designed a method for acquiring immediate-autopsy specimens of the Eustachian tube in which RNA was well preserved for in situ hybridization and Northern analysis (27). This newly developed method was used for characterization of mucin gene expression in the Eustachian tube of humans.

The middle ear and Eustachian tube are integral parts of the auditory system and are involved in sound...
transduction from the tympanic membrane to the inner ear. Proper functioning of the auditory system is reliant on the mucociliary transport system, which originates in the inferior tympanum and covers the entire Eustachian tube (5), evacuating invading microorganisms and clearing the middle ear cavity. This maintains an air- and pressure-balanced environment, free of infection, for the sound-conducting structures (the ossicular chain). The function of the mucociliary transport system is largely dependent upon interactions between mucins, cilia, and periciliary fluid.

It is clear from this study that the Eustachian tube possesses a profile of MUC5B, MUC5AC, MUC4, and MUC1 mucins. Secretory MUC5B and MUC5AC mucins are major structural components of the mucociliary transport system in the Eustachian tube under noninflamed conditions. Membrane-bound MUC1 and MUC4 mucins may play a role in cellular protection in the Eustachian tube. Because MUC4 is expressed in ciliated, intermediate, and basal cells within the entire epithelium and contains epidermal growth factor-like domains, it may be involved in protein-protein interaction and play a role in cellular renewal of the epithelium. MUC4 has been shown to act as a ligand and modulator for the receptor tyrosine kinase ErbB2 (8, 9), which is involved in cellular growth and proliferation. It is still unknown, however, how mucins in the Eustachian tube are altered by inflammation, as none of the eight Eustachian tubes harvested for this study had evidence of otitis media.

The lining of the middle ear cavity is thought to be a modified respiratory epithelium, a transitional epithelium ranging from squamous cells at the superior tympanum to columnar cells at the inferior tympanum. A ciliated tract rings the tympanic orifice and extends up to the inferior promontory area. Goblet cells are distributed in the ciliated tract under normal conditions. This study demonstrated that some cells in the promontory area of the middle ear were labeled with the MUC5B mucin gene probe, suggestive of MUC5B mucin productivity. The primary mucin involved in the mucociliary transport system of the middle ear appears to be MUC5B. MUC5AC was not detectable in the middle ear, nor were MUC4, MUC1, or other MUCs detectable in the noninflamed ears.

The middle ear has a unique or modified mucin gene expression pattern when compared with that of the Eustachian tube or airway. Tracheobronchial epithelium mainly expresses MUC5AC (4, 33) and weakly expresses MUC5B (4); Eustachian tube epithelium expresses MUC5AC and MUC5B equally, whereas middle ear epithelium expresses mainly MUC5B. There appears to be an alteration in the mucin pattern of the epithelium as it ascends from the airway to the middle ear; expression of MUC5AC is weakened or profoundly downregulated in epithelium as it ascends from the airway to the middle ear.

MUC5B and MUC5AC mucins share regions of oligonucleotide similarity at their 3'- and 5'-ends, and their DNA loci are close to each other (at 11p15.5) and believed to be derived from the same ancestral gene. The functional significance of this double distribution of secretory mucins in the Eustachian tube is not clear.

### Table 3. Summary of mucin mRNA and glycoproteins in the Eustachian tube and middle ear

<table>
<thead>
<tr>
<th>MUCs</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
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ET, Eustachian tube (noninflammatory); ME, middle ear (noninflammatory); ME*, inflammed middle ear; ISH, in situ hybridization; NB, Northern blot; IHC, immunohistochemistry; MUCs, mucins; M1, MUC1; M2, MUC2; M3, MUC3; M4, MUC4; M5AC, MUC5AC; M5B, MUC5B; M6, MUC6; M7, MUC7; M8, MUC8.
MUC5B has the largest central tandem repeat region (13) among known mucins. Because the adhesive portion of mucin is within the central area (tandem repeat units), one would expect that the length of the tandem repeat region would be a contributing factor toward efficient entrapment and adhesion to pathogens. It was suggested in a von Willebrand factor molecule study that tandem repeat units originated to catch moving molecules or objects (11).

Secreted middle ear mucin is a branched, chainlike strand interspersed with bulblike portions (Fig. 6). The linear portion of the strand may represent the tandem repeat region of mucin, which is heavily glycosylated and therefore rigid. The “bulbs” may represent non-tandem repeat regions that are less glycosylated and rich in cysteine residues, likely areas in which disulfide bonds could link inter- and intramolecularly between mucin monomers to form mucous strands.

The amino acid sequence of MUC5B deduced from cDNA indicates abundant cysteine residues at both ends of the molecule (25). Some disulfide bonds between MUC5B mucin monomers were noted to survive reduction with DTT in this study, indicative of protected disulfide bonds between MUC5B monomers. Dimerization of mucin monomers occurs at the endoplasmic reticulum (2, 12); however, it is not clear where dimerized mucins polymerize. On the basis of the biochemical properties, structural characteristics, and gene organization, it is highly plausible that MUC5B in the human body is an extremely viscous mucin, characteristic of a high density (1.4 g/ml, a very large peptide backbone (8,000 amino acids) with abundant carbohydrates), gel-forming capability (a chainlike strand capable of further linkage), and resistance to reduction (remaining polymerized after DTT treatment). It would be of interest to know whether MUC5B mucin accumulation in the middle ear cavity is a major cause of “glue ear,” a type of otitis media in which mucus gel plugs the middle ear.

This study indicated that MUC5B mucin is a major structural component of the mucociliary transport system that maintains homeostasis of the middle ear. Under inflamed conditions, MUC5B and MUC4 mucins were highly upregulated in the middle ear in association with an increase in MUC5B/MUC4 mucin-producing cells (Fig. 3, h and k), suggestive of a transition from normal middle ear epithelium to a hypersecretory epithelium. Because MUC5B and MUC4 are evident in the middle ear effusion, it is clear that they contribute to formation of mucoid effusion, which can cause disruption of the mucociliary transport system and conductive hearing loss. MUC4 may participate in proliferation and differentiation of middle ear epithelial cells, as suggested in other studies (6). In this study, MUC5B mucin gene expression in the middle ear was not shown to be limited to goblet cells under inflamed conditions; it appeared that the entire middle ear epithelium is involved in MUC5B mucin production (Fig. 4A, M5B, d). Two recent studies that reported MUC5B to be present in middle ear epithelium and effusion in patients with COM (23, 24) support these findings. Therefore, highly active production of MUC5B and MUC4 mucins in the middle ear mucosa and accumulation in the middle ear cavity of mucins capable of forming a mucus gel may be key to compromised middle ear function and resultant clinical disease.

The mechanism for upregulation of MUC5B and MUC4 in middle ear epithelium is not currently understood, since the promoter area of these mucin genes has not been studied. Also, the factors that upregulate these mucin genes in the middle ear with otitis media have not been identified. It is essential to investigate the molecular mechanisms responsible for upregulation of MUC5B and MUC4 mucins and mucus hyperproduction. Insight into these mechanisms will facilitate the development of pharmaceutical intervention strategies against mucus hyperproduction in COM or MOM.

We thank JoAnn Knox for editing the manuscript.

This work was supported in part by National Institute on Deafness and Other Communication Disorders Grant R01-DC-03433, the Lions 5M Multiple District Hearing Foundation, and the Veterans Affairs Research Service.

REFERENCES