Otopathogenic *Pseudomonas aeruginosa* Strains as Competent Biofilm Formers

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**Objective:** To determine whether *Pseudomonas aeruginosa*, a common cholesteatoma pathogen, known to form biofilms in other chronic infections, is capable of contributing to biofilm formation in cholesteatoma.

**Design:** We tested 12 OPPA isolates for several aspects of biofilm formation, including adherence to human keratinocytes, expression of quorum-sensing genes, twitching motility, and production of extracellular matrix as determined by both crystal violet staining and carbazole reaction.

**Results:** Ten OPPA strains demonstrated increased adherence (1.5- to 12-fold) to human keratinocytes relative to PAO1, a laboratory strain. Expression of *las* and *rhl* quorum-sensing products were detected in 11 OPPA strains. By crystal violet staining, we found biofilm formation in all OPPA strains equal to or greater than that found in PAO1 (2- to 18-fold). In addition, OPPA strains demonstrated mucoid characteristics, including down-regulation of twitching motility and increased alginate production.

**Conclusions:** Strains of OPPA isolated from cholesteatoma are strongly adherent to keratinocytes and capable of forming biofilm. In addition, OPPA strains have mucoid characteristics in vitro. When these bacteria assume a biofilm phenotype, they are highly resistant to antibiotics and host defenses. These data suggest that OPPA can contribute to biofilm formation in cholesteatoma, leading to the persistence of this infection.


**MANY CHRONIC OTOLOGIC** infections are characterized by their resistance to antibiotics. Aural cholesteatomas often become chronically infected, leading to more aggressive disease and increased resistance to nonsurgical treatments. Resistance to antimicrobial agents and host defenses are hallmarks of microbial biofilms. Our recent findings of dense bacterial colonies embedded in extracellular matrix within the keratin debris of cholesteatomas led to the hypothesis that this chronic ear infection may be a consequence of biofilm formation. An understanding of the underlying infectious process and the role of microbial biofilms in cholesteatomas is necessary for the further development of nonsurgical adjuvant therapies to eradicate persistent and recurrent infections.

*Pseudomonas aeruginosa* (PA) is the organism most commonly isolated from aural cholesteatoma, representing 25% to 35% of total isolates. In addition, PA is widely studied because of its propensity to form biofilms under a variety of conditions and in diseases where biofilm formation is suspected. Most notably, studies have implicated the PA biofilm phenotype in the chronicity and antibiotic resistance of the chronic pulmonary infection seen in cystic fibrosis (CF).

If cholesteatoma infections are a biofilm disease, then at least 1 of the infecting species of bacteria should be capable of biofilm formation. Since PA is the most common clinical isolate from cholesteatoma infections, we hypothesized that PA strains isolated from cholesteatoma would demonstrate a biofilm phenotype.

Twelve strains of otopathogenic PA (OPPA) were isolated from infected cholesteatomas and evaluated for characteristics that should affect the development of a mature biofilm in cholesteatomas, including the ability of the organism to adhere to a surface, communicate via quorum sensing, demonstrate twitching motility, and produce extracellular matrix. The hypothesis that PA is a biofilm-forming pathogen in cholesteatoma is supported if OPPA isolates display these requisite biofilm forming capabilities.
Table 1. Primers for Quorum-Sensing RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>lasR</td>
<td>5'-GTCTCAAGGAAAAGGCAG</td>
<td>5'-ATGGCGAAGAAAAGGCTGAGTT</td>
</tr>
<tr>
<td>lasI</td>
<td>5'-AGGAAGCTTGAATCTGAAG</td>
<td>5'-GCAAGACCTGTGCTGCCTTC</td>
</tr>
<tr>
<td>rhlI</td>
<td>5'-GCTGACAGCTTCTTGAGAT</td>
<td>5'-TTTGCAGGGTTGCTTTC</td>
</tr>
<tr>
<td>rhlR</td>
<td>5'-TTTGCAGGGTTGCTTTC</td>
<td>5'-AGAGATAGGCAGATTTC</td>
</tr>
<tr>
<td>16s</td>
<td>5'-GAGGAAGGGTGGAAGCTG</td>
<td>5'-AGGCAGGGGAAAGTATTAC</td>
</tr>
</tbody>
</table>

Abbreviation: RT-PCR, reverse transcriptase–polymerase chain reaction. *We include the 16s gene for comparison.

**METHODS**

**INITIAL SPECIMEN COLLECTION AND ORGANISM IDENTIFICATION**

Cholesteatoma bacterial specimens were collected after surgical excision from 70 patients and by sterile swabs of clinically infected cholesteatomas in 17 nonsurgical candidates. Cholesteatoma specimens collected after surgical excision were incubated overnight in tryptic soy broth at 37°C. Bacterial samples from ear swabs were plated on a nonselective Luria-Bertani (LB) plate. Gram staining was performed to identify aerobic bacteria. If no organisms were visualized, no further characterization was undertaken.

**HUMAN KERATINOCYTE CULTURES**

Primary adult normal human epidermal keratinocytes (NHEK; Clonetics, San Diego, Calif) were cultured on 6-well cell culture plates. Keratinocytes were initially seeded at 100,000 cells/cm² and allowed to reach confluency by maintaining the cell culture with keratinocyte growth medium 2 (KGM-2; Clonetics) at a 0.15 mM calcium concentration. Terminal differentiation was achieved by incubating the keratinocyte monolayer in KGM-2 at a 1.0 mM calcium concentration for 2 days.

**TRANSFORMATION OF OPPA**

Plasmid pMRP9-1 containing a green fluorescent protein (GFP) cassette in pUCP-1810 was introduced into each PA strain by electroporation, as previously described.12 Briefly, bacteria were electroporated at 1800 V in an Electroporator 2510 (Eppendorf North America, Westbury, NY), incubated in LB at 37°C, while being rotated at 250 rpm for 1 hour, and plated onto selective media (carbenicillin at 300 µg/mL) (Sigma, St Louis, Mo). The bacterial suspension was mixed with 100 µL of 1 mM MOPS (3-morpholinopropane sulfonic acid) at a 1:100 volume/volume ratio. Biofilm phase cultures were grown under nonshaking conditions at 37°C in 6-well polystyrene plates for 48 hours. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, Calif) followed by the manufacturer’s protocol. Total RNA (1.0 µg) was digested with RNase-free DNase I (Roche, Indianapolis, Ind). Complementary DNA (cDNA) synthesis was performed using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche).

The RT-PCR reaction was as follows; 1 X PCR buffer, 0.2 mM dNTPs (deoxyribonucleotide triphosphates; Roche), 1.5 mM magnesium chloride, primers (16s, 1.0 µM; experimental, 5.0 µM) and 0.1 U of Taq polymerase (Invitrogen). Reactions were performed on a PTC-100 (MJ Research Inc, Waltham, Mass). Products were separated on a 2.0% agarose gel at 100 mV for 1 hour, stained with ethidium bromide (Sigma), and visualized under UV illumination on a Gel Doc 2000 Gel documentation system (BioRad).

**TWITCHING MOTILITY**

Twitching motility was assayed as previously described.13 Briefly, each strain was stab inoculated into a 1.5% agar LB plate. After 24 hours of incubation at 37°C, the zone of hazy diameter was measured. The OPPA strains were grown from a glycerol stock of the initial pass of the isolated PA strain to minimize phenotypic changes that might occur with repeated passage.

**CRYSTAL VIOLET BIOFILM ASSAY**

Biofilm formation was assayed by crystal violet staining as previously described.14 Briefly, 100 µL of a 1:100 dilution of the attached PA was washed 3 times with phosphate-buffered saline to remove unbound bacteria. Subsequently, the keratinocytes were trypsinized, and the number of adherent bacteria was determined by colony count. Fluorescent microscopic images of Pseudomonas keratinocyte adhesion was obtained by growing keratinocytes on a glass-bottomed well. Vybrant cell labeling solution and DAPI (+’6’-diamidino-2-phenylindole) nucleic acid stain (Molecular Probes, Eugene, Ore) were used to stain the cell membrane and nucleus, respectively, using the manufacturer’s protocol. The GFP-labeled OPPA strains at 10⁶ bacteria/mL were then exposed to the fluorescently stained keratinocytes for 1 hour. Images were acquired using a Nikon Eclipse TE2000U microscope (Tokyo, Japan) at high power under oil with the following filters: 358/461 nm for DAPI; 595/615 nm for Vybrant; and 494/518 nm for GFP. Images were overlaid using AnalySIS software (Soft Imaging, Lakewood, Colo).

**QUORUM-SENSING GENES las AND rhl**

Primers for lasR, lasI, rhlR, and rhlI were designed for reverse transcriptase–polymerase chain reaction (RT-PCR) using primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All gene sequences were derived from the sequence of PAO1, a laboratory strain, available at www.pseudomonas.com. Primers were synthesized by Integrated DNA Technologies (Skokie, Ill) (Table 1). Bacterial seed cultures were grown overnight from a single colony in LB media at 37°C while rotating at 250 rpm. The seed culture was inoculated into fresh LB broth at a 1:100 volume/volume ratio. Biofilm phase cultures were grown under nonshaking conditions at 37°C in 6-well polystyrene plates for 48 hours. Total RNA was isolated using TRIZOL Reagent (Invitrogen, Carlsbad, Calif) following the manufacturer’s protocol. Total RNA (1.0 µg) was digested with RNase-free DNase I (Roche, Indianapolis, Ind). Complementary DNA (cDNA) synthesis was performed using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche).

The RT-PCR reaction was as follows; 1 X PCR buffer, 0.2 mM dNTPs (deoxyribonucleotide triphosphates; Roche), 1.5 mM magnesium chloride, primers (16s, 1.0 µM; experimental, 5.0 µM) and 0.1 U of Taq polymerase (Invitrogen). Reactions were performed on a PTC-100 (MJ Research Inc, Waltham, Mass). Products were separated on a 2.0% agarose gel at 100 mV for 1 hour, stained with ethidium bromide (Sigma), and visualized under UV illumination on a Gel Doc 2000 Gel documentation system (BioRad).

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was added at a 1:5 dilution and incubated at room temperature for 15 minutes. The wells were then washed 5 times with sterile deionized water. To elute the crystal violet, 95% ethanol was added to each well and a 100-µL aliquot was removed and placed in a 96-well microtiter plate. The amount of crystal violet eluted was read using a microplate reader (λ = 595 nm) (Biotek Synergy HT, Winooski, Vt).

### ALGINATE QUANTIFICATION ASSAY

Alginate production was quantified by measuring the uronic acid component of alginate with a modified carbazole reaction adapted from Poschet et al. A mucoid PA strain (PA-CF) isolated from a pediatric patient with CF and obtained from the St Louis Children's Hospital Clinical Microbiology Laboratory served as a positive control. In brief, PA strains were grown on Pseudomonas isolation agar supplemented with 20% glycerol for 72 hours at 37°C. The bacteria were scraped from the plate and resuspended in 10 mL of sterile 0.85% sodium chloride. The suspension was centrifuged at 7000 g for 10 minutes. After the supernatant was transferred, wet bacterial weight was determined, and 350 µL of the supernatant was layered on 3 mL of uronic acid detection reagent (2.93 mL of sulfuric acid and 70 µL of borate solution containing 45 mM potassium hydroxide and 1M boric acid). Then 0.1% weight/volume carbazole (Sigma) in ethanol was added to the mixture and incubated at 55°C for 30 minutes. The amount of alginate was measured spectrophotometrically at 530 nm against a standard curve of alginic acid (Sigma) and expressed as micrograms of alginate per milligram of wet bacterial weight.

### STATISTICAL ANALYSIS

Statistical analysis was performed using Sigma Stat, version 3.0 by t test and analysis of variance (α = .05) (SPSS Science, Chicago, Ill).

### RESULTS

Strains of PA were collected from a total of 87 human cholesteatoma specimens over a 2-year period. In 30 of the 87 specimens, bacteria were not detected by gram stain after an overnight incubation, and no further analysis was performed. Gram-positive cocci infected 26 specimens, with coagulase-negative Staphylococcus species accounting for most of these infections. Twenty-three cholesteatomas were infected with gram-negative bacilli. Pseudomonas aeruginosa was identified in 12 specimens (21%), the highest incidence of any gram-negative organism (Table 2). No significant differences were seen in the bacterial isolates between surgical excision and ear swabs.

### ADHERENCE OF OPPA TO PRIMARY HUMAN KERATINOCYTE MONOLAYERS

Cholesteatomas are primarily composed of keratin debris and terminally differentiated keratinocytes. To determine if OPPA strains could adhere to keratinocytes and potentially initiate cholesteatomas infections, we exposed terminally differentiated keratinocytes to PA at a multiplicity of infection of approximately 100 bacteria per keratinocyte for 2 hours.

### QUORUM-SENSING GENE PRODUCTS

Ten of 12 OPPA strains demonstrated increased adherence to keratinocytes relative to the laboratory strain PAO1. This increased adherence was statistically significant and varied from 150% (OPPA3) to 1240% (OPPA11) that of PAO1 (P < .05). The OPPA1 strain demonstrated adherence equal to that of PAO1; however, OPPA5 was significantly less adherent than PAO1 (Figure 1). An additional laboratory strain, PAK, demonstrated significantly less adherence to keratinocytes (approximately 0.2%) relative to PAO1 and all OPPA strains (P < .05) (data not shown). Thus, we concluded that OPPA can adhere to human keratinocytes in vitro. This suggests that OPPA is also capable of binding to the surface of cholesteatomas.

### RT-PCR OF QUORUM-SENSING GENE PRODUCTS

The formation of a biofilm by PA is regulated in a cell density–dependent manner via a cell-to-cell communication system referred to as quorum sensing. Pseudomonas aeruginosa uses 2 N-acyl homoserine lactone (AHL)-dependent quorum-sensing systems, the las and rhl systems. We used RT-PCR to detect the expression of lasR, lasI, rhlR, and rhlI by OPPA strains in a biofilm phase. Ribonucleic acid was isolated after PA strains had established a visual biofilm in a nonshaking culture after 48 hours. Eleven of the 12 OPPA strains expressed lasR, lasI, rhlR, and rhlI. We did not detect any of these quorum-sensing products in OPPA1 (Figure 2). These results suggest that most OPPA strains use the 2 known quorum-sensing systems, las and rhl, to establish biofilms. The identification of OPPA1 as PA was verified by independent analysis performed by the St Louis Children’s Hospital Clinical Microbiology Laboratory.

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Table 2. Bacteriology of Cholesteatoma

<table>
<thead>
<tr>
<th>Organism</th>
<th>Specimens Isolated, No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-positive cocci</strong></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>8 (14.0)</td>
</tr>
<tr>
<td>Coagulase-negative staphylococci</td>
<td>17 (29.8)</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td><strong>Gram-positive bacilli</strong></td>
<td></td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td><strong>Gram-negative bacilli</strong></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>12 (21.1)</td>
</tr>
<tr>
<td>Achromobacter xylosidans</td>
<td>3 (5.2)</td>
</tr>
<tr>
<td>Alcaligines species</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td><strong>Gram-negative bacilli</strong></td>
<td></td>
</tr>
<tr>
<td>Bacillus fragilis</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Enterobacter cloaceae</td>
<td>2 (3.5)</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Stentrophomonas maltophilia</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Untyped†</td>
<td>3 (5.2)</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>2 (3.5)</td>
</tr>
<tr>
<td>Sterile</td>
<td>30</td>
</tr>
</tbody>
</table>

†More than 4 organisms grew out and were not separated for organism identification.

*Gram-negative bacilli that did not grow sufficiently for organism identification.

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TWITCHING MOTILITY

The type IV pili of PA have been shown to be important for the adherence and subsequent colonization of eukaryotic cells.\(^{15}\) In addition, type IV pili are required for surface-associated motility, termed twitching motility.\(^{11}\)

Despite the evidence supporting the role of type IV pili in the initiation of infection, PA obtained from patients with CF and chronic pulmonary infections demonstrate a decreased type IV pili twitching motility.\(^{16}\) All strains of OPPA except OPPA9 demonstrated statistically significant decreases in twitching motility relative to PAO1. Furthermore, 3 OPPA strains (OPPA2, OPPA5, and OPPA7) demonstrated no twitching motility. OPPA9 showed twitching motility equal to that of PAO1 (\(P < .05\)). Based on the model of a chronic biofilm infection seen in CF pneumonia, this decrease in twitching motility is consistent with bacteria recovered from a chronic biofilm infection.\(^{16}\) This finding suggests that OPPA strains were isolated from a chronic biofilm state and supports the hypothesis that cholesteatomas are biofilm infections.

CRYSTAL VIOLET QUANTIFICATION OF BIOFILM FORMATION

By crystal violet staining, all OPPA strains are capable of equivalent or enhanced biofilm formation relative to PAO1 after a 24-hour nonshaking incubation at 37°C (Figure 4). Ten of the 12 OPPA strains exhibited 2- to 18-fold greater biofilm formation than PAO1 by this assay (\(P < .05\)). The OPPA7 and OPPA10 strains were roughly equal to PAO1 in biofilm formation under these conditions.
ALGINATE PRODUCTION

The extracellular matrix that characterizes PA biofilms is composed of a combination of polysaccharides, nucleic acids, and proteins. A carbazole reaction was used to quantify alginate production in all PA strains, including a mucoid PA strain (PA-CF) isolated from a pediatric patient with CF, which served as a positive control. Using this method, 11 of the 12 OPPA strains (all strains except OPPA12) demonstrated statistically significant increases in alginate production relative to PAO1 (P<.05). In addition, 8 of the 12 OPPA strains formed alginate in amounts equal to or greater than PA-CF (P<.05) (Figure 5).

COMMENT

Chronic infections of the ear are among the many infectious processes being reevaluated in light of increased recognition of the role of biofilms in clinical disease. Most recalcitrant aural infections are associated with entrapped keratin within the middle ear or mastoid (cholesteatoma), a favorable culture medium for bacteria. Despite the relative accessibility of the ears to topical antimicrobials and oral antibiotics, these infections often persist. In some cases, thorough surgical debridement combined with antimicrobial therapy is unable to prevent the recurrence of the infection with the same organism. In addition, recalcitrant otitis media infections can also progress to otitis media with effusion, an infection characterized by resistance to antibiotics. The formation and persistence of bacterial biofilms may, in part, account for these 2 processes. The characteristic feature of the biofilm phenotype is increased resistance of bacteria to antibiotics and host defenses, which provides a mechanism for the persistence of the chronic infection in otitis media with effusion and cholesteatoma.

If OPPAs contribute to biofilm formation in cholesteatoma, these organisms should be capable of developing a mature biofilm. Biofilms can be defined as communities of microorganisms encased in a self-produced extracellular matrix and adherent to a surface. Although the processes used by PA to produce a mature biofilm are not fully understood, it is thought that adhesion to a surface, followed by intercellular communication via quorum sensing, altered twitching motility, and production of extracellular matrix are involved. We have demonstrated that PA is a common cholesteatoma organism that displays properties consistent with the formation of biofilms. These data indicate that cholesteatoma is a biofilm infection and that OPPA strains are biofilm-forming organisms that may contribute to the persistence of these infections.

An early step in establishing a biofilm infection is the adherence of the bacteria to a surface. Otopathogenic strains of PA demonstrate increased adherence to terminally differentiated keratinocytes compared with PAO1. This increased adherence varied from 150% (OPPA3) to 1240% (OPPA11) that of PAO1. Other studies have demonstrated increased adherence of certain strains of PA to external auditory canal epithelium. These results further suggest that a potential mechanism of increased...
pathogenicity in these clinical isolates may be enhanced adhesion to keratinocytes. It is possible that increased adhesion by OPPA may be a result of specific mechanisms developed to adhere to keratinocytes. Although the bacterial structures that mediate the adherence of PA to keratinocytes are currently unknown, there are some candidates. The initial adherence of PA to pneumocytes is mediated by type IV pili that bind to the asialo-GM(1) receptor of respiratory epithelium. Recent evidence suggests that PA may also use other host receptors and non-type IV plus adhesion genes such as cupA, a fimbrial adhesin, to mediate adherence to other surfaces.22,23

Intercellular signaling is critical for coordinating biofilm formation and its associated changes in gene expression. Pseudomonas aeruginosa uses 2 quorum-sensing pathways, las and rhl, to sense changes in cell density that regulate biofilm formation and the production of virulence factors. LasR and RhlR direct the synthesis of freely diffusing autoinducers. LasR and RhlR are receptors for these autoinducers and serve as transcriptional activators of the system at high cell densities. The expression of quorum-sensing genes by most OPPA strains is consistent with their production of biofilms and their potential to act as aural pathogens. The single OPPA strain that did not yield detectable las and rhl products, OPPA1, nonetheless produces a biofilm, as demonstrated by crystal violet staining and alginate production. The OPPA1 strain may coordinate biofilm formation by an unknown quorum-sensing system or may contain sequence polymorphisms within the las and rhl systems that prevent PCR amplification with our primers.

It is significant that each strain of OPPA is able to form a biofilm in equal measure to PAO1, a well-established biofilm-forming strain. Most OPPA strains show significantly greater amounts of crystal violet staining than PAO1. The presence of dense colonies of bacteria within the matrix of the cholesteatoma and the ability of OPPA strains to form biofilm in vitro are consistent with ability of OPPA strains to establish biofilms in vivo.

In addition to exhibiting a biofilm-forming phenotype, OPPA also demonstrates mucoid characteristics. The down-regulation of twitching motility and the increased production of alginate in OPPA strains compared with PAO1 are consistent with a mucoid phenotype. OPPA also demonstrates mucoid characteristics. OPPA1 and OPPA2 have equal alginate production, OPPA2 has significantly greater biofilm formation under crystal violet evaluation (P<.05). These findings are consistent with the heterogeneous nature of the components of the PA extracellular matrix.18

Herein we have shown that PA is a common pathogen of chronic cholesteatoma infections. Otopathogenic PA strains demonstrate increased adherence to keratinocytes and increased biofilm formation by crystal violet staining compared with PAO1. In addition, OPPA exhibits mucoid characteristics that may correlate to the presence of a chronic infection. The characterization of OPPA as biofilm-forming strains that also demonstrate mucoid characteristics supports the notion that cholesteatoma and chronic ear infections are likely biofilm diseases.

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REFERENCES


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**Announcement**

**Trial Registration Required**

As a member of the International Committee of Medical Journal Editors (ICMJE), Archives of Otolaryngology—Head & Neck Surgery will require, as a condition of consideration for publication, registration of all trials in a public trials registry (such as http://ClinicalTrials.gov). Trials must be registered at or before the onset of patient enrollment. This policy applies to any clinical trial starting enrollment after July 1, 2005. For trials that began enrollment before this date, registration will be required by September 13, 2005, before considering the trial for publication. The trial registration number should be supplied at the time of submission.

For details about this new policy, and for information on how the ICMJE defines a clinical trial, see the editorial by DeAngelis et al in the June issue of Archives of Otolaryngology—Head & Neck Surgery (2005;131:479-480). Also see the Instructions to Authors on our Web site: www.archoto.com.