Short Communication

Pathogenesis of Middle Ear Cholesteatoma

A New Model of Experimentally Induced Cholesteatoma in Mongolian Gerbils

Tomomi Yamamoto-Fukuda,^{*,†}
Yoshitaka Hishikawa,[†] Yasuaki Shibata,[‡]
Toshimitsu Kobayashi,[§] Haruo Takahashi,^{*}
and Takehiko Koji[†]

From the Departments of Otolaryngology-Head and Neck Surgery, Translational Medical Science, Histology and Cell Biology,[†] and Oral Pathology and Bone Metabolism,[‡] Nagasaki University Graduate School of Biomedical Sciences, Nagasaki; and the Department of Otorhinolaryngology, Head and Neck Surgery,[§] Tohoku University School of Medicine, Sendai, Japan*

Middle ear cholesteatoma is characterized by enhanced proliferation of epithelial cells with aberrant morphological characteristics. To investigate the origin of the cholesteatoma cells, we analyzed spontaneously occurring cholesteatomas associated with a new transplantation model in Mongolian gerbils (gerbils). Cholesteatomas were induced in gerbils with a transplanted tympanic membrane by using the external auditory canal (EAC) ligation method. After the pars flaccida of the tympanic membranes were completely removed from male gerbils, corresponding portions of tympanic membranes of female gerbils were transplanted to the area of defect, and then we ligated the EAC (hybrid-model group). As a control group, the EAC of normal male and female gerbils was ligated without myringoplasty. In all ears of each group, the induced cholesteatomas were seen. *In situ* PCR was then performed to detect the mouse X chromosome-linked *phosphoglycerate kinase-1* (*pgk-1*) gene on the paraffin sections. One *pgk-1* spot in the epithelial nuclei was detected in male cholesteatoma, and two *pgk-1* spots were detected in female cholesteatoma, respectively. On the other hand, in the hybrid-model group, we detected not only one but also two *pgk-1* spots in the epithelial nuclei of cholesteatoma. These results strengthened the evidence that the origin of epithelial cells in cholesteatoma is the tympanic membrane in this model, but not the residential mid-

dle ear epithelial cells or the skin of the EAC. (*Am J Pathol* 2010; 176:2602–2606; DOI: 10.2353/ajpath.2010.091182)

Middle ear cholesteatoma is a pathological condition associated with otitis media,^{1,2} accompanying hearing loss, and occasionally facial palsy,³ and recurrence after surgical treatment is very common.⁴ Untreated or advanced cholesteatoma can also lead to more serious complications such as skull bone erosion, brain abscess, and meningitis.⁵ Middle ear cholesteatoma is morphologically characterized by epithelial cell proliferation and granulation tissue formation; unfortunately, however, our understanding of the molecular mechanism underlying the pathogenesis of cholesteatoma is only limited. Despite many studies, the exact pathogenesis of middle ear cholesteatoma has not been fully understood.

In 1999 Kim and Chung⁶ detected that the expression of epithelial markers in cholesteatoma was similar to that of the epidermis of the external auditory canal (EAC) and that epithelial cells of cholesteatoma have different characteristics than those of the tympanic membrane. These findings suggest a relationship between cholesteatoma and EAC, as suggested by Ruedi and other investigators.^{7,8}

On the other hand, there is clinical evidence for the “retraction and proliferation theory” on the pathogenesis of cholesteatoma. Sudhoff and Tos⁹ suggested that the proliferation of the epithelial cells in the retraction pocket will be altered by inflammatory stimuli of the subepithelial connective tissue and finally that may lead to cholesteatoma formation.¹⁰ But there is a lack of explanation for the transition from a retraction pocket to cholesteatoma.¹⁰

Supported in part by Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports, and Culture (number 1247003) and by a grant from the Japanese Environment Agency (to T. Koji).

Accepted for publication March 1, 2010.

Address reprint requests to Tomomi Yamamoto-Fukuda, M.D., Department of Otolaryngology-Head and Neck Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. E-mail: tomomiyf@nagasaki-u.ac.jp.

Figure 1. A diagram of the EAC technique. **A:** EAC-ligated ear of the hybrid model. A tympanic membrane that was excised from a female (XX) gerbil was implanted into a male (XY) gerbil's tympanic membrane in the ear. **B:** EAC-ligated ear of male gerbil is shown. **C:** EAC-ligated ear of female gerbil is shown. **Arrows:** XX, female cells; XY, male cells.

Animal models are very important for studying the pathogenesis of aural cholesteatoma.^{11–13} Mongolian gerbils (gerbils), *Meriones unguiculatus*, have a propensity for the development of aural cholesteatoma.¹¹ In addition to developing spontaneously with aging, cholesteatomas can be experimentally induced via EAC ligation in the gerbil.¹²

To investigate the origin of the epithelial cells of cholesteatoma, whether from the epithelial cells of EAC or tympanic membrane, we made a new model of experimentally induced cholesteatoma in gerbils and use a new analysis system named *in situ* PCR.¹⁴

Materials and Methods

Biochemical and Chemicals

Paraformaldehyde (PFA) was purchased from Merck (Darmstadt, Germany). Proteinase K was from Wako Pure Chemicals (Osaka, Japan). Cy-3 was from Amersham Biosciences Corporation (Piscataway, NJ). TaKaRa Ex Taq (TaqDNA polymerase, deoxynucleotid mixture, 10 × PCR buffer, and MgCl₂) was from Takara Bio, Inc. (Shiga, Japan). 3-Aminopropyltriethoxysilane, bovine serum albumin, yeast tRNA, salmon testis DNA, digoxigenin-dUTP, and Brij 35 were purchased from Sigma Chemical Co. (St. Louis, MO). Formamide was purchased from Nakarai (Kyoto, Japan). All other reagents used in this study were purchased from Wako Pure Chemicals or Sigma Chemical Co. and were of analytical grade. A Slow Fade Light Antifade kit was obtained from Molecular Probes (Eugene, OR).

Animals and Tissue Preparation

The study was conducted in 12-week-old gerbils. All experiments were conducted according to the principles and procedures outlined in the Guidelines for Animal Experimentation of Nagasaki University with the approval of the

Institutional Animal Care and Use Committee. Five male and five female gerbils with normal eardrums were used as the hybrid model. Each animal was anesthetized with pentobarbital intraperitoneally. After we performed tympanic membrane perforation in the right ear of male gerbils, we performed a myringoplasty by way of an overlay technique; the ossicular chain was intact in gerbils and no ossiculoplasty was done. Tympanic membrane that was excised from female gerbil was implanted into male gerbil's perforation of tympanic membrane in the right ear (Hybrid model). One month follow-up was obtained, and the graft take rate was 100% without otorrhea, perforation, or effusion. An animal model of cholesteatoma was induced in gerbil with the EAC ligation method (Figure 1, A–C).¹² The right ear canals of each gerbil were ligated with 4-0 silk suture through a curvilinear incision behind the pinna (Hybrid model group, Figure 1A). As controls, five male and five female gerbils (12 weeks old) with normal eardrums and without myringoplasty were used, and right EAC was ligated to make experimental cholesteatoma (control group, Figure 1B and C). The ligature was placed medially, as close to the bony meatus as possible to reduce variability. At 2 years after ear canal ligation, animals were anesthetized by diethyl-ether and transcardially perfused with 0.01M PBS, pH 7.2, followed by 4% PFA in PBS (pH 7.4). The temporal bones were removed *en bloc* and fixed with 4% PFA/PBS at 4°C for 18 hours. After fixation, the temporal bones were decalcified by EDTA at 4°C for 7 days¹⁵ and embedded in paraffin. Sections (5-μm thickness) were prepared and then mounted on 3-aminopropyltriethoxysilane-coated glass slides. Adjacent sections were stained with H&E and other sections were processed for *in situ* PCR.

Hybridization Probe and PCR Method

Genomic DNA was prepared from the testis or ovary of gerbils with AquaPure Genomic DNA Kits (Bio-Rad, Richmond, CA) according to the instruction manual provided by the manufacturer. When the PCR conditions were

optimized, the band intensity corresponding to the increased amount of DNA correlated linearly with the PCR cycle number. The bands were quantitated by densitometry (NIH ImageJ software, version 1.08i, <http://rsb.info.nih.gov/ij/>, last accessed on August 25, 2009), and each value was expressed relative to that of gerbil glyceraldehyde-3-phosphate dehydrogenase. Initial detection of the X chromosomes was achieved by simultaneous amplification of X-linked *phosphoglycerate kinase locus* (*pgk-1*).^{16,17} All primer sequences were synthesized commercially and, apart from *pgk-1*, sequences were obtained from Research Genetics Inc. (Huntsville, AL). The 170 bp products from the mouse X-linked *pgk-1* locus were amplified by using forward and reverse primers (BEX Co., Tokyo, Japan) 5'-CACGCTTCAAAGCGCACGTCT-3' and 5'-CTTGAGGGCAGCAGTACGGAAT-3', respectively, whereas primers (BEX Co.) corresponding to mouse *glyceraldehyde-3-phosphate dehydrogenase* were 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. PCR method was performed as previously reported.¹⁸ The Gene Amp PCR System 9400 (PE Applied Biosystems, Foster City, CA) was used for gene amplification. PCR was performed in a 20- μ l final volume of reaction mixture, containing 0.5 units of TaKaRa Extaq polymerase, 0.2 mmol/L of each deoxynucleotid mixture, ExTaq buffer (1 $\times$), 1 μ mol/L of each specific primer described above, and 100 ng of testis or ovary DNA as a template. Each cycle of PCR included 15 seconds of denaturation at 94°C, 15 seconds of annealing at 55°C, and 40 seconds of extension at 72°C. After 28 reaction cycles, the PCR products with *pgk-1* primers and glyceraldehyde-3-phosphate dehydrogenase primers were loaded onto a 3% agarose gel, and the DNA bands of the predicted size were cut, purified with glass powder, and terminator cycle sequencing was determined with ABI

PRISM 310 Genetic Analyzer (PE Applied Biosystems) by using the instruction provided by the supplier.

In Situ PCR

To analyze the localization of *pgk-1* gene in paraffin sections, *in situ* PCR was performed according to the method of Hishikawa et al.¹⁸ The oligo-DNAs that can amplify part (170 bp) of the mouse X chromosome-linked *pgk-1* gene were used as primers as described above. The tissue sections were deparaffinized and rehydrated by using standard procedures. These sections were digested with 50 μ g/ml of proteinase K (37°C, 15 minutes). After post-fixation with 4% PFA in PBS (5 minutes), the sections were washed in PBS and immersed in 50% formamide in 2 $\times$ standard saline citrate (1 $\times$ standard saline citrate = 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) overnight at 4°C. After washing with double-distilled water (5 minutes, three times), the sections were completely desiccated. For each slide, the amplification mixture was prepared in a final volume of 100 μ l, containing 1 $\times$ PCR buffer, 1.0 μ g/ml forward primer, 1.0 μ g/ml reverse primer, 0.2 mmol/L dNTP, 2.5 mmol/L MgCl₂, and 2.5 U/100 μ l TaqDNA polymerase. To label the amplified DNA, 2.0 μ mol/L Cy-3 was used. The slides were sealed with EasiSeal (Hybaid, Ashford, UK) and then placed on the heated plate of the OmniSlide System (Hybaid); the DNA in the tissues then was denatured at 94°C for 3 minutes. As for PCR cycle, 25 cycles of amplification (denaturation for 15 seconds at 94°C, annealing for 15 seconds at 56°C, extension for 60 seconds at 72°C) were run. The slides were then heated at 72°C for 5 minutes to extended DNAs. After amplification, the coverslips were

Figure 2. H&E stainings in a combination of an experimental surgical method with spontaneous development cholesteatoma and normal ear in the gerbils. **A:** Cholesteatoma was induced in all ears of gerbils by the EAC ligation method, despite myringoplasty. Cholesteatoma appear as cyst-like structures lined with the stratified squamous epithelium (E) of the tympanic membrane and ear canal with thick subepithelium (S) and filled with keratin (K). These cholesteatoma had enlarged into the bulla and filled the bulla completely. **B:** Higher magnification of cholesteatoma (see inset in A) is shown. **Arrows** indicate the bone resorption is occurring at the advancing front of the cholesteatoma. **C:** The bulla of the normal gerbiline ear contained a thin layer of simple squamous epithelium (E) with thin subepithelium (S). **D:** Higher magnification of normal ear (see inset in C) is shown.

removed, and the slides were washed with $2 \times$ standard saline citrate (37°C , 15 minutes, four times). To confirm the specificity of signals, a variety of control experiments were conducted. In every experiment, as a negative control, *in situ* PCR was done omitting TaqDNA polymerase or the primers. Furthermore, some sections were immersed with random primers to provide definitive evidence for the sequence specificity of the signal.

Visualization and Image Analysis

The sections were covered with Slow Fade Light Antifade kit solution and viewed through the fluorescence-microscope equipped with the 560 nm band of UV laser. Cy-3 signals were detected by using a Zeiss fluorescence microscope connected to a CCD camera (AxioCam, Carl Zeiss, Jena, Germany).

Results

PCR Products

PCR products comprising 170 bp were obtained and were confirmed by DNA sequencing to be part of the *pgk-1* gene. Semiquantitative analysis of these products using the ImageJ analysis system showed that the quantity of the *pgk-1* gene products from the ovary was about twofold more than that from the testis.

Cholesteatoma Formation

When examined 2 years after canal ligation, cholesteatoma formations occurred spontaneously in five of five right ears and bilaterally in five of the left ears. The results of H&E staining were that eight of ten cholesteatomas had enlarged into the bulla and filled the bulla completely (Figure 2, A–D), and one of them had cholesteatoma that eroded into the middle and posterior cranial fossae (Figure 2B).

Identification of Epithelial Cells of Origin by *In Situ* PCR

In the hybrid model group, one or two *pgk-1* spot(s) were detected in the epithelial nuclei of cholesteatomas (Figure 3A) and one *pgk-1* spot was detected in the cells of EAC (Figure 3B). On the other hand, in the control group, one *pgk-1* spot in the nuclei of epithelial cells of cholesteatoma was detected in male (Figure 3C), and one or two *pgk-1* spot(s) were detected in female cholesteatomas (Figure 3D), respectively.

The number of one or two *pgk-1* spot(s) cells in each group were as follows (Figure 3E). In the hybrid model, we detected not only one *pgk-1* spot in eight epithelial cells, but also two *pgk-1* spots in 27 epithelial cells of cholesteatoma tissue. On the other hand, only one *pgk-1* spot was detected in 54 cells of the epidermis of EAC. In the control group, one *pgk-1* spot was detected in 39 epithelial cells in male cholesteatoma. And one *pgk-1* spot was de-

Figure 3. *In situ* PCR in hybrid model and control. **A:** One or two *pgk-1* spot(s) were detected in the epithelial nuclei of cholesteatoma. **B:** On the other hand, one *pgk-1* spot was detected in the cells of EAC. Original magnification, $\times 350$. **C:** One *pgk-1* spot in the nuclei of epithelial cells was detected in male cholesteatoma. **D:** One or two *pgk-1* spot(s) were detected in female cholesteatoma, respectively. **Arrows** indicate two spots and **arrowheads** indicate one spot. **E:** The number of cells having one or two X chromosome(s). In the hybrid model group, we detected one *pgk-1* spot in eight epithelial cells and two *pgk-1* spots in 27 epithelial cells of cholesteatoma tissue. On the other hand, only one *pgk-1* spot was detected in 54 cells of the epidermis of EAC. In the control group, one *pgk-1* spot was detected in 39 epithelial cells in male cholesteatoma, and one *pgk-1* spot was detected in 17 epithelial cells or two *pgk-1* spots were detected in 37 epithelial in female cholesteatomas. Black bars indicate numbers of one X chromosomal cells and white bars indicate numbers of two X chromosomal cells.

tected in 17 epithelial cells, or two *pgk-1* spots were detected in 37 epithelial cells in female cholesteatoma.

Discussion

Using a newly created animal model named “local hybrid ear model,” we were able to consistently induce cholesteatomas and to analyze the origin of cholesteatoma-inducing epithelial cells by using gender discrimination of cells in tissue sections from gerbils. *In situ* PCR of cholesteatoma tissues confirmed the female origin of the tympanic epithelial cells that were transplanted into male recipients and identified the tympanic membrane as the probable source of the epithelium associated with cholesteatomas.

In the present study, we used the number of X chromosomes to discriminate male (XY) from female (XX) cells. To trace the X chromosome, we chose *pgk-1* as a

marker gene because the gene structure is well analyzed and it is strictly linked with the X chromosome.¹⁹ However, standard *in situ* hybridization is not sensitive enough to mark a specific chromosome with a single gene in a tissue section and thus the more sensitive *in situ* PCR method was used. In the context, it should be noted that *in situ* PCR has not exactly succeeded to gain wide popularity, partly because of a significant fluctuation in the optimal conditions of the protocol depending on tissues and primer. Therefore, we first optimized thoroughly the *in situ* PCR protocol for X-linked *pgk-1* gene by using ovarian and testis paraffin sections.¹⁸ Indeed, in our previous study, we detected one to four and one to two spots of *pgk-1* in the nuclei of granulosa and germ cells, respectively.¹⁸

The EAC-ligated model of gerbilline cholesteatomas has been considered as the most reliable one inducing experimental cholesteatoma, and believed to be useful in studying the pathophysiology of cholesteatomas, because of a consistency of bilateral cholesteatoma and the similarity to human cholesteatomas. In addition to these advantages, this animal model allows for manipulation of cells between the sexes and provides a method to track the origin of the gender of cellular components by using the presence of a single versus double X chromosome marker. On the other hand, our hybrid model has some disadvantages such as requirement for the skill to perform successful surgery and long time to develop cholesteatomas. Although we can save the time by using cholesteatomatous cell *in vitro* culture, we think *in vivo* study is more appropriate to understand the pathogenesis of cholesteatoma, because the interaction between the epithelial cells and inflamed subepithelial stromal cells is important in the cholesteatoma formation.¹⁰

In a paraffin section, cells are supposed to contain a whole nucleus or a part of nucleus. In this context, we must be sure that there are two kinds of cells containing one *pgk-1* spot. One is the cell that has XY chromosomes, and the other is the cell that has XX chromosomes. The percentage of the number of cells having one *pgk-1* spot or two *pgk-1* spots of cholesteatoma tissue in our hybrid model was almost the same as that of female cholesteatoma. These results indicated that the cells having one *pgk-1* spot of cholesteatoma tissue have XX chromosome.

Using *pgk-1* as a tracer, we have provided evidence that transplanted tympanic membranes were the origin of the epithelial cells associated within development of age-related spontaneous bilateral cholesteatomas in gerbils. Residential middle ear epithelial cells or the skin of the external ear canal could be eliminated as a source of the cholesteatoma inducing epithelium because the functions of these cells are different. In fact, the previous study revealed the different protein and cytokeratin profiles between cholesteatoma keratinocytes and epithelial cells of middle ear.^{20,21} The results strongly demonstrated the evidence that the origin of epithelial cells in cholesteatoma is the tympanic membrane, but not residential middle ear epithelial cells or the skin of the external ear canal, in this hybrid model of cholesteatoma. And also our results might indicate a possibility that squamous metaplasia of tympanic mem-

brane is considered to be an adaptive tissue response toward persistent pathological stimuli such as chronic inflammation and possible etiopathogenetical mechanism for middle ear cholesteatoma.

References

1. Harker LA: Cholesteatoma an evidence study. Cholesteatoma First International Conference. Edited by BF McCabe, J Sade, M Abramson. Birmingham, Aesculapius Publications, 1977, pp 308–309
2. Tos M: Incidence, etiology and pathogenesis of cholesteatoma in children. *Adv Otorhinolaryngol* 1988, 40:110–117
3. Bagger-Sjoberg D, Phelps PD: Cholesteatoma with extension to the cochlea. *Am J Otol* 1985, 6:338–343
4. Edelstein DR, Parisier SC: Surgical techniques and recidivism in cholesteatoma. *Otolaryngol Clin North Am* 1989, 22:1029–1040
5. Chole RA: Cholesteatoma conference summary. Cholesteatoma and Mastoid Surgery the Fourth International Conference. Edited by Y Nakano. Amsterdam, Kugler Publications, 1992, pp. 835–840
6. Kim CS, Chung JW: Morphologic and biologic changes of experimentally induced cholesteatoma in Mongolian gerbils with anticytokeratin and lectin study. *Am J Otol* 1999, 20:13–18
7. Ruedi L: Pathogenesis and surgical treatment of middle ear cholesteatoma. *Acta Otolaryngol* 1979, 361:1–45
8. Park KH, Chun YM, Park HJ: Cytokeratin immunohistochemistry of acquired cholesteatoma in middle ear. *Korean J Otolaryngol Head Neck Surg* 1994, 37:5–13
9. Sudhoff H, Tos M: Pathogenesis of attic cholesteatoma: clinical and immunohistochemical support for combination of retraction theory and proliferation theory. *Am J Otol* 2000, 21:786–792
10. Yamamoto-Fukuda T, Aoki D, Hishikawa Y, Kobayashi T, Takahashi H, Koji T: Possible involvement of keratinocyte growth factor and its receptor in enhanced epithelial-cell proliferation and acquired recurrence of middle-ear cholesteatoma. *Lab Invest* 2003, 83:123–136
11. Chole RA: Cholesteatoma: spontaneous occurrence in the Mongolian gerbil, *Meriones unguiculatus*. *Am J Otol* 1981, 2:204–210
12. McGinn MD, Chole RA, Henry KR: Cholesteatoma: experimental induction in the Mongolian gerbil, *Meriones unguiculatus*. *Acta Otolaryngol* 1982, 93:61–67
13. Wolfman DE, Chole RA: Experimental retraction pocket cholesteatoma. *Ann Otol Rhinol Laryngol* 1986, 95:639–644
14. PCR 3. PCR in situ hybridization: a practical approach. The Practical Approach Series Number 186. Edited by CS Herrington, JJ O'Leary. Oxford University Press, New York, 1997
15. Yamamoto-Fukuda T, Shibata Y, Hishikawa Y, Shin M, Yamaguchi A, Kobayashi T, Koji T: Effects of various decalcification protocols on detection of DNA strand breaks by terminal dUTP nick end labeling. *Histochem J* 2000, 32:697–702
16. Adra CN, Boer PH, McBurney MW: Cloning and expression of the mouse *pgk-1* gene and the nucleotide sequence of its promoter. *Gene* 1987, 60:65–74
17. Singer-Sam J, Chapman V, LeBon JM, Riggs AD: Parental imprinting studied by allele-specific primer extension after PCR: paternal X chromosome-linked genes are transcribed prior to preferential paternal X chromosome inactivation. *Proc Natl Acad Sci USA* 1992, 89:10469–10473
18. Hishikawa Y, An S, Yamamoto-fukuda T, Shibata Y, Koji T: Improvement of *in situ* PCR by optimization of PCR cycle number and proteinase K concentration: localization of X chromosome-linked phosphoglycerate kinase-1 gene in mouse reproductive organs. *Acta Histochem Cytochem* 2009, 42:15–21
19. Goto M, Koji T, Mizuno K, Tamaru M, Koikeda S, Nakane PK, Mori N, Masamune Y, Nakanishi Y: Transcription switch of two phosphoglycerate kinase genes during spermatogenesis as determined with mouse testis sections *in situ*. *Exp Cell Res* 1990, 186:273–278
20. Olszewska E, Sudhoff H: Comparative cytokeratin distribution patterns in cholesteatoma epithelium. *Histol Histopathol* 2007, 22:37–42
21. Xu Y, Tao ZZ, Hua QQ, Wang XX, Xiao BK: Expression and activation of nuclear factor-kappaB in middle ear cholesteatoma. *Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi* 2006, 41:455–459