Identification of *Id1* in Acquired Middle Ear Cholesteatoma

Quan-An Zhang, MD; Yuki Hamajima, MD; Qing Zhang, MD; Jizhen Lin, MD

**Objectives:** To determine (1) the relationship between chronic inflammatory changes in the ossicular chain area (OCA) and the formation of cholesteatoma and (2) the correlates between aberrant gene expression and abnormal proliferation of cholesteatoma.

**Methods:** Two hundred sixty-four ears with chronic otitis media that had undergone ear surgery were included in this study for statistical analysis of the relationship between abnormalities in the OCA and cholesteatoma. Fourteen middle ear cholesteatoma specimens were collected for immunohistochemical analysis of candidate molecules involved in the abnormal proliferation of keratinocytes. A cell model was used for verification of candidate molecule involvement.

**Results:** The formation of cholesteatoma was accompanied by chronic inflammatory changes in the OCA, including granulated tissue, adhesion, and stagnating effusion. The inhibitor of the DNA-binding (*Id1*) gene, which is involved in controlling cell cycle progression, was abundantly expressed in cholesteatoma epithelium. In vitro studies indicate that *Id1* regulated the expression of nuclear factor κB, cyclin D1, proliferating cell nuclear antigen, and cell cycle progression of keratinocytes.

**Conclusions:** Chronic inflammation in the OCA is closely related to the formation of cholesteatoma. The *Id1*/nuclear factor κB/cyclin D1/proliferating cell nuclear antigen signaling pathway is involved in the abnormal proliferation of keratinocytes in acquired cholesteatoma.


**Author Affiliations:**
Departments of Otolaryngology, Xi’an Jiao Tong University, Xi’an, People’s Republic of China (Drs Q.-A. Zhang and Q. Zhang), Nagoya City University, Nagoya, Japan (Dr Hamajima), and Otitis Media Research Center, University of Minnesota, Minneapolis (Dr Lin).
lesteatoma. Finally, we conducted in vitro studies to confirm the importance of ild1 in the pathogenesis of cholesteatoma.

Formation of the retraction pocket in the pars flaccida and posterosuperior quadrant of the tympanic membrane in the clinical patients was accompanied by the chronic inflammatory changes in the OCA, including granulated tissue, adhesion, and stagnating effusion. Ild1 and its effectors were abundantly expressed in the cholesteatoma epithelium. In vitro, ild1 stimulated the cell cycle progression and growth of cultured keratinocytes via the activation of nuclear factor κB (NF-κB), cyclin D1, and proliferating cell nuclear antigen (PCNA).

PROCUREMENT OF SURGICAL SPECIMENS
Clinical patients who had undergone ear surgery were recruited from Jiao-Tong University Medical School, Xi’an, China. The study was performed in compliance with institutional human subject study regulations. The study included 26+ ears of clinical patients with chronic OM. Diagnosis of cholesteatoma was made clinically and confirmed pathologically after surgery. The criteria for retraction pocket and cholesteatoma are as follows: (1) a tympanic membrane foosette or a tympanic membrane that is profoundly concave toward the middle ear cavity, and (2) onionskin-like substances accumulated in the foosette or middle ear cavity. For the studies of cholesteatoma and inflammatory location, the middle ear cleft was divided into 3 areas: (1) the anterior hemimesotympanum area (AHMA, the area before vertical plane via the tympanic umpiculus); (2) the OCA, including the attic and posterosuperior part of the mesotympanum; and (3) the antrum-mastoid process area (AMPA). Special attention was paid to the relationship between the pathologic changes in the OCA and the formation of retraction pockets and cholesteatomas. The differences in pathologic changes among the above-mentioned 3 areas were evaluated by t test, with a P value of less than .05 being considered significant. Fourteen surgical cholesteatoma specimens from 11 patients were collected from the University of Minnesota Hospitals and Clinics, Minneapolis, and Ichinomiya City Hospital, Nagoya, Japan, for histopathologic and immunohistochemical analysis, to investigate the location of the pathologic changes, especially in the OCA of the middle ear cleft, and the expression of ild1 and its effector proteins: NF-κB, cyclin D1, PCNA, and keratin 10. Four normal skin specimens from clinical patients was recruited from Jiao-Tong University Medical School, Xi’an, China. The study was performed in compliance with institutional human subject study regulations.

IMMUNOHISTOCHEMICAL ANALYSIS
Surgical cholesteatoma specimens were fixed in 10% formalin, dehydrated in an alcohol series, embedded in paraffin, cut into 5-µm sections, deparaffinized in xylene, and incubated with primary antibodies (anti-ild1 antibody, Santa Cruz Biotechnology Inc, Santa Cruz, California; keratin 10, NeoMarkers, Lab Vision Corp, Fremont, California; “activated” NF-κB and MAB3026, Chemicon International Inc, Temecula, California; cyclin D1, BD Biosciences, San Jose, California; and PCNA, Abcam Inc, Cambridge, Massachusetts) and secondary antibodies sequentially conjugated to fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate (TRITC), using protocols previously described.7

CONSTRUCTION OF ild1 COMPLEMENTARY DNA
Full-length ild1 complementary DNA (cDNA) was cloned into a protein-expressing vector (pEGFP; Clontech, Mountain View, California) using standard protocols. Briefly, the open reading frame of the ild1 gene was obtained by reverse transcription–polymerase chain reaction using the following primer pair (5’-AGCCAGTCGCAAGAATCATG-3’/5’-TTGCCCCTG-GATGCGTGGG-3’) and cloned into the vector according to the manufacturer’s instructions. ild1 cDNA in the vector (hereafter referred to as ild1) was confirmed by sequencing. An empty vector was used as a control.

CELL CYCLE PROGRESSION
Human keratinocytes (Rhek-1A)8 were maintained in Eagle minimal essential medium (Invitrogen Corp, Carlsbad, California) supplemented with 10% fetal bovine serum, penicillin/streptomycin (50 µg/mL), and 2mM l-glutamine (hereafter referred to as full-growth medium [FGM]) at 37°C in a 5% carbon dioxide air atmosphere. During transient transfection of cells, cells were cultured in T-75 flasks with FGM up to 60% confluence and then incubated with serum-free medium (Opti-MEM; Invitrogen Corp) supplemented with 6 µg/mL of hexadimethrine bromide (Polybrene; Invitrogen Corp) (hereafter referred to as transfection medium) for 16 hours, recovered in FGM for 24 hours, and harvested for evaluation of flow cytometry and cell cycle progression.

FLOW CYTOMETRY
The cells were cultured in T-75 flasks with FGM until they reached 60% confluence, transfected with either ild1 or empty vectors at 1.4 µg/mL in transfection medium for 16 hours, recovered in FGM for 24 hours, and harvested for fluorescence-activated cell sorting by a flow cytometer (FACSCalibur; BD Biosciences). Briefly, they were incubated with 0.3% saponin in phosphate-buffered saline (PBS) for 5 minutes, incubated with antiactivated NF-κB for 30 minutes on ice, washed twice with 0.3% saponin in PBS, incubated with TRITC-conjugated goat anti–mouse secondary antibody for 30 minutes on ice, washed twice with 0.3% saponin in PBS, and analyzed with the flow cytometer with a commercially available cell analysis software system (CellQuest Pro; BD Biosciences). For double-labeling flow cytometry, the cells were incubated first with a primary antibody (cyclin D1), followed by a corresponding secondary antibody (conjugated to TRITC) and then with a second primary antibody (PCNA) followed by corresponding secondary antibody (conjugated to allopheocyanin) in a similar way. Cells incubated with nonspecific IgG (Santa Cruz Biotechnology Inc) followed by TRITC-or Cy3-conjugated secondary antibodies served as controls. A total of 10,000 cells per sample were analyzed with the cell analysis software system (CellQuest Pro) using red, green, and/or blue channels for detection of TRITC, green fluorescent protein, and/or Cy5 fluoresceins, respectively. Graphic data were analyzed with flow cytometry data analysis software (FlowJo, 7.1 Version; Tree Star Inc, Ashland, Oregon) and photoshop software (Version 7.0; Adobe Systems Inc, San Jose, California). Experiments were run in triplicate. The results are presented as the percentage of positive cells for ild1- and empty vector–transfected cells after the subtraction of nonspecific IgG background.


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progression assay by flow cytometer. Briefly, the cells were washed with PBS and incubated with propidium iodide at 1 mg/mL for 15 minutes. A total of 20,000 cells per sample were analyzed with the cell analysis software system (CellQuest Pro). Graphic data were analyzed as described in the “Flow Cytometry” section. The results are presented as the percentage of proliferating cells (G2/M phase) over total cells.

RESULTS

Histologic and immunohistochemical examinations were performed on the 14 surgical cholesteatoma tissue specimens from clinical patients. The epithelial layers of retraction pocket cholesteatoma specimens were positive for antibody to Id1 (Figure 1A) and keratin 10 (Figure 1B, top layer of cholesteatoma epithelium). To locate the expression positions relative to one another, double labeling was performed. It was demonstrated that Id1 was extensively expressed in the entire mucosal layer of middle ear cholesteatoma tissues and that keratin 10 was mainly located in the top mucosal layer (Figure 1C). Of the 14 specimens studied, 12 were positive for both Id1 and keratin 10 and 2 were positive for keratin 10 but negative for Id1 (the epithelial layer was missing and only the onionskinlike layer was visible). In normal middle ear mucosa specimens, the epithelial layer was negative for both Id1 and keratin 10 but the submucosal blood vessels were positive for Id1 (data not shown). In normal neck skin epithelium, Id1 was barely detectable in the basal cells, and Id1 signals were located in the nuclei (Figure 1F). Id1 also was coexpressed with activated p65 (a heterodimer of NF-κB, Figure 1I). Cyclin D1 and PCNA were expressed in the basal layer of cholesteatoma epithelium (Figure 1D and E).

Fluorescent-activated cell sorting demonstrated that Id1 cDNA was successfully transfected into the Rhek-1A cells, with approximately 63.4%±7.4% (mean±SD) cells (n=3) being positive for green fluorescent protein in Id1 cDNA- or empty vector–transfected cells. On cell cycle analysis, Id1 significantly increased the G2/M phase cells over controls (42.5%±3.4% vs 36.5%±3.7%, P<.05). Id1 transfection significantly increased activated NF-κB expression compared with empty vector transfection (66.8%±4.2% vs 5.9%±1.2%, P<.05), cyclin D1 expression compared with empty vector transfection (14.9%±2.1% vs 5.2%±1.3%, P<.05), and PCNA expression compared with empty vector transfection (9.5%±1.3% vs 5.2%±0.8%, P<.05). Id1 increased the coexpression of cyclin D1 and PCNA compared with empty vector transfection (8.3%±1.5% vs 1.6%±0.3%). Representative graphic data are presented in Figure 2.

Among the clinical patients, cholesteatomas were found in 33 ears (15 in the left ear, and 18 in the right ear; 19 men and 14 women). Cholesteatoma of the middle ear cavity occurred significantly more often in the OCA than in the AHMA (P<.05). The percentages of granulation and cholesteatoma tissue, respectively, in the 3 areas of the middle ear were as follows: AHMA, 0% and 15.1%; OCA, 81.8% and 100%; and AMPA, 60.4 and 90.9%. The ossicular chain was frequently destroyed or missing (>90%) or partially destroyed by cholesteatoma and...
granulation tissue in the OCA. In 264 ears of clinical patients with chronic OM, the percentages of persistent effusion and granulation tissue, respectively, in the 3 areas of the middle ear were as follows: AHMA, 11.0% and 0.8%; OCA, 83.7% and 90.5%; and AMPA, 91.3% and 93.0%; the incidence of persistent effusion and granulation tissue was much higher in the OCA than in the AHMA (P < .05).

Because of aggressive and expanding growth, cholesteatoma and granulation tissue simultaneously occupied the OCA, the AHMA, and the AMPA. Also, there was overlapping of effusion and granulation tissue in the 3 areas of the middle ear.

**COMMENT**

The chronic inflammatory changes in the OCA are more severe than those in the other 2 areas of the middle ear. It is difficult to drain the OCA owing to its structural features, so effusion tends to stagnate in that area. The anatomical drain passage from the OCA to the inferior mesotympanum is narrow and readily blocked by the swelling and adhering mucosa due to the mucosal folding and the tympanic isthmus infrastructure in the OCA. Our early data indicate that granulation tissue develops only in the area where the effusion stagnates, suggesting that chronic inflammation in the OCA is irritating. In contrast, pathologic changes in the AHMA, such as congestion, edema, and effusion, are usually temporary and reversible, which explains why almost no cholesteatoma originates from the central area of the pars tense of the tympanic membrane or from the mesotympanum. We believe that the inflammatory infiltration and irritation of the external squamous layer of the pars flaccida and posterosuperior quadrant of the tympanic membrane play an important role in the development of retraction pockets and subsequent cholesteatomas.

Chronic inflammation in the OCA may activate the expression of Id1 in the basal cells. Id1 then triggers the cellular growth and proliferation process of the basal cells via NF-κB/cyclin D1/PCNA signaling. In previous stud-
In the skin, Id1 up-regulation is associated with highly proliferating cells such as squamous cell carcinoma or is involved in the immortalization of keratinocytes. Keratinocytes at the basal layer of cholesteatomal epithelium with the Id1 protein expression are considered to be “activated” or “transformed” because they possess a self-growth capability and have a tendency to recur after removal. Together with the up-regulation of NF-kB and PCNA, Id1 is considered an important mitogen for aggressive growth of keratinocytes in middle ear cholesteatoma. Because Id1 is one of the major effector molecules in the transforming growth factor signaling pathway that is actively involved in tissue remodeling and fibrotic disorders, it is quite possible that Id1 plays a role in the process of middle ear granulation tissue formation and subsequent cholesteatoma.

In summary, chronic inflammatory changes in the OCA were accompanied by the formation of retraction pockets and cholesteatomas in which epithelial tissues were positive for the expression of Id1 protein, which is associated with NF-kB, cyclin D1, and PCNA. The in vitro studies indicate that Id1 regulates NF-kB, cyclin D1, and PCNA, which elicit cellular growth and proliferation of keratinocytes, suggestive of a disease mechanism for middle ear cholesteatoma in humans.

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Correspondence: Jizhen Lin, MD, Department of Otolaryngology, University of Minnesota, 2001 Sixth St SE, Lions Research Bldg, Room 216, Minneapolis, MN 55455 (linxx004@umn.edu).


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