Cell Proliferation and Apoptosis in Human Middle Ear Cholesteatoma

Hiromi Kojima, MD; Yasuhiro Tanaka, MD; Toshihide Tanaka, MD; Hidemi Miyazaki, MD; Masanori Shiwa, MD; Yosuke Kamide, MD; Hiroshi Moriyama, MD

Objective: To compare the pattern of proliferation and apoptotic cell death in cholesteatoma tissues with that in normal skin.

Participants: The cholesteatoma tissue samples were excised from 10 patients during surgery. Normal skin specimens collected from the external ear canal of 6 of the 10 patients were used as controls.

Results: In all cholesteatoma tissue samples, apoptotic cells were not seen in the basal cell layer, but they were observed in the suprabasal, prickle, and granular cell layers. In skin specimens obtained from normal external ear canal skin, in which the suprabasal cell layer was comparatively small, similar kinetics of apoptotic cell death were observed. Immunohistochemical analysis using a monoclonal antibody to proliferation cell nuclear antigen demonstrated the presence of proliferating cells in the basal and suprabasal cell layers of the normal external ear canal skin, whereas in the cholesteatoma tissue samples, large numbers of proliferation cell nuclear antigen–positive cells were also observed in the prickle and granular cell layers.

Conclusions: Proliferation in cholesteatoma epidermal cells is not uncontrolled, as it is in malignant tumors. Our results demonstrate an increase in the rate of proliferation and apoptotic cell death in cholesteatoma epidermis.


Development as well as maintenance of many adult tissues is achieved by a dynamic balance among cell proliferation, differentiation, and programmed cell death. Cells are eliminated by apoptosis, which is genetically encoded cell death defined by characteristic, morphological, and biochemical changes.1-4

The histopathological characteristics of cholesteatoma are hyperproliferation and differentiation of the epithelium and accompanying destruction of the bones. In cholesteatoma, the accumulation of keratinous debris has been shown mainly to result from a blockage in horizontal keratin transport. Recent studies have shown that the expression of c-jun and p53 proteins is increased in human cholesteatoma.5 It was postulated that these proteins may have role in keratinocyte differentiation, hyperproliferation, and apoptosis.5 However, the precise mechanism responsible for the regulation of cholesteatoma growth is unclear.

The present study was undertaken to compare the rate of proliferation and apoptotic cell death in cholesteatoma tissue samples with that in tissue samples of normal external ear canal skin. Proliferation cell nuclear antigen (PCNA), a nonhistone nuclear protein expressed in the late G1, S, and G2 phases of the cell cycle, was used as a marker for proliferation.6 An in situ apoptosis detection system was used to detect digoxigenin-labeled genomic DNA of apoptotic cells. Our results demonstrate an increase in rate of proliferation and apoptotic cell death in cholesteatoma epidermis.

RESULTS

IN SITU LABELING OF APOPTOTIC CELLS

We looked for apoptotic cell death in tissue specimens of cholesteatoma excised from 10 patients and compared the results with those of normal external ear canal skin specimens from 6 of the same 10 patients. In situ labeling of apoptotic cells detected several positive cells in the suprabasal, prickle, and granular cell layers of cholesteatoma tissue sections. No
## PATIENTS AND METHODS

### TISSUES

Our study comprised 10 patients with pars flaccida-type middle ear cholesteatoma who underwent middle ear surgery at Jikei University School of Medicine, Tokyo, Japan, from December 1994 through November 1995. The patients (5 men and 5 women) ranged in age from 33 to 56 years (mean age, 43 years). Specimens of normal external ear canal skin were also obtained from 6 of the 10 patients. The excised specimens were immediately immersed in 10% buffered formalin solution and were fixed overnight at room temperature. The specimens were embedded in paraffin, sliced into 3- to 4-μm sections, and mounted on poly-l-lysine-coated slides.

### IN SITU LABELING OF APOPTOTIC CELLS

Apoptosis was detected by labeling the 3′OH ends of DNA using digoxigenin incorporation by terminal deoxynucleotidyl transferase. Antidigoxigenin antibodies and immunoperoxidase staining were used to demonstrate digoxigenin-nucleotide incorporation with a commercially available in situ apoptosis detection system (Apop Tag, Oncor, Gaithersburg, Md). In brief, paraffin-embedded sections were deparaffinized and dehydrated in graded alcohol and treated with 20-μg/mL proteinase K at room temperature for 15 minutes. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in phosphate-buffered saline. After digoxigenin-nucleotide was added catalytically, detection with anti–digoxigenin peroxidase was performed using 3,3′-diaminobenzidine for 6 minutes. The tissue sections were visualized after counterstaining with 1% methyl green.

### ANTIBODY

The PCNA monoclonal antibody (PC-10) used in this study is commercially available (Dakopatts, Glostrup, Denmark).

### PCNA IMMUNOHISTOCHEMICAL STAINING

Slides were deparaffinized and dehydrated in graded alcohol. The deparaffinized sections were soaked in 0.01-mol/L citrate buffer and heated in a microwave oven for 10 minutes at 650 W,7 followed by blocking of endogenous peroxidase with 0.3% hydrogen peroxidase in methanol. Nonspecific binding was blocked with 10% normal horse serum and 1% bovine serum albumin for 20 minutes. After a brief rinse, the sections were immunoreacted with a PCNA monoclonal antibody (1:300) at room temperature for 30 minutes. The sections were washed in phosphate-buffered saline, incubated for 1 hour with biotinylated anti-mouse IgG (1:100), and then treated with avidin-biotin complex for 1 hour; 3′-diaminobenzidine was then applied as a chromogen (Vector Labs Inc, Burlingame, Calif). Sections were visualized after counterstaining with 1% methyl green.

The PCNA labeling and apoptotic index were estimated by the percentage of cells scored under a light microscope at a 200-fold magnification. A minimum of 200 cells were counted in 2 different areas of each section. The Student t test was used to evaluate the labeling and apoptotic indexes for statistical significance.

Apoptotic cells were observed in the basal cell layer (Figure 1). The pattern and kinetics of the apoptotic cells in the normal external ear skin specimens were similar (Figure 2). The quantitative analysis of apoptotic cell death was carried out by counting the live and dead cells in at least 3 different areas of the sections. In the cholesteatoma epidermis, 66.8±7.8% (mean±SD) of the cells showed apoptotic characteristics (P<.001). Similar results (59.5±10.1%) were observed in tissue sections from normal external ear canal skin. There was no statistical difference in the apoptotic index between normal external ear canal skin specimens and cholesteatoma tissues.

### PCNA IMMUNOHISTOCHEMICAL STAINING

Immunohistochemical analysis using anti–PCNA antibody demonstrated large numbers of proliferating cells in the basal, suprabasal, and prickle cell layers of all cholesteatomatous tissues (Figure 3). However, in all 6 specimens from normal external ear canal skin, the PCNA-positive cells were observed predominantly in the basal and suprabasal cell layers. Occasional cells were positive for PCNA in the prickle cell layer, but cells of the granular cell layer were all negative (Figure 4). The quantitative analysis demonstrated that in contrast to the PCNA-positive cells (10.2±2.4%) in specimens from normal external ear canal skin, the cholesteatoma epidermal cells demonstrated a high proliferative rate (74.9±10.0%; P, not significant). Statistical analysis also showed that there was a significant increase in the proliferative rate in cholesteatoma tissues when compared with the normal external ear canal skin samples.

### COMMENT

The mechanism of the hyperproliferative state of cholesteatoma epidermis is still unknown. Previous studies have shown that the epidermal growth factor receptor messenger RNA is expressed only in the basal cell layer of normal external ear canal skin, while its expression is increased in all epidermal layers of cholesteatoma.8 The fact that epidermal growth factor receptor messenger RNA is expressed in the prickle and upper cell layers of cholesteatoma suggests the possibility that the detached cells from the basal cell layer are still immature and retain the ability to undergo cell proliferation.

Other studies have shown that CK16, a cytokeratin that is overexpressed in psoriasis, solar keratosis, and squamous cell carcinoma, is involved in proliferation. Studies on cholesteatoma epidermis have also demonstrated a higher expression of CK16.9,10 In a study based on uptake of tritiated thymidine, cholesteatoma epidermis was shown to have a proliferation profile similar to that of a...
malignant tumor. Recent studies have shown an increase in the expression of Ki-67, a protein that is involved in proliferation. These findings suggest that many factors may contribute to the hyperproliferative characteristics of cholesteatoma.

The present study demonstrated the presence of PCNA-positive cells in the basal and suprabasal cell layers of skin tissues from normal external ear canals. However, increased PCNA expression corresponding to hyperproliferative states was observed in the prickle cell layer in cholesteatoma tissue specimens. Tsuruhara et al also found an increase in PCNA-positive cells in the prickle cell layer in cholesteatoma tissue specimens.

Comparing the immunohistochemical pattern of PCNA expression of normal external ear canal skin with that of cholesteatoma tissues, we demonstrated that in cholesteatoma the suprabasal and upper layer cells, as well as the basal cells, have the ability to undergo cell division. In the samples of normal external ear canal skin, the process of differentiation begins after the cells are detached from the basal layer. We postulate that in cholesteatoma there could be some abnormality in the differentiation process, resulting in the proliferation of detached cells. However, the proliferation in cholesteatoma epidermal cells is not uncontrolled, as it is in malignant tumors. Our results demonstrate that despite the hyperproliferation in the cholesteatoma epidermis, cells retain the capability to undergo apoptotic cell death. However, in malignant tumors, cells not only divide uncontrollably but they also become resistant to apoptotic cell death. Even though the rate of apoptotic cell death in cholesteatoma is the same as that in normal external ear canal skin, there is a significant increase in the number of dead cells in cholesteatoma owing to hyperproliferation in the epidermal cell layers. Besides the accumulation of keratinous debris due to a blockage in horizontal keratin transport, increased cell death will also contribute to increased keratinous debris.

Recent studies have shown that the expression of wild-type p53 protein is increased in keratinocytes in the prickle cell layer of cholesteatoma epidermis. Tumor-suppressor gene p53 has been shown to be involved in the regulation of the cell cycle and in apoptosis. Increased expression of p53 in the prickle cell layer of cho-
lesteatoma epidermis might contribute to the increase in apoptotic cell death. However, more work is required to clarify the role of apoptotic cell death in cholesteatoma.

Accepted for publication October 8, 1997.
Reprints: Hiromi Kojima, MD, Department of Otorhinolaryngology, Jikei University School of Medicine, 3-25-8 Nishishinbashi Minatoku, Tokyo 105, Japan.

REFERENCES