Role of Bcl-xL Protein in Differentiation and Apoptosis of Human Middle Ear Cholesteatoma Epithelium

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Objective: To compare the mechanisms of proliferation, differentiation, and apoptosis in middle ear cholesteatoma epithelium with those of normal external ear canal epithelium.

Design: The localizations of the expression of Bcl-xL protein and involucrin and the presence of apoptotic cells were determined for tissue slices of middle ear cholesteatoma epithelium and compared with the findings for normal external ear canal epithelium. In addition, SCC-25/bcl-xL transfectants showing the overexpression of Bcl-xL were used to investigate the effect of this protein on the expression of involucrin, which is a marker of epithelial cell differentiation.

Materials: Cholesteatoma tissue specimens were surgically excised from 10 patients. Normal skin specimens collected from the external ear canal of the 10 patients were used as control specimens.

Results: The expression of Bcl-xL was detected in the vicinity of the basal cell layer of both the cholesteatoma epithelium and the normal external ear canal epithelium. Conversely, the expression of involucrin (ie, a marker of epithelial cell differentiation) increased in proportion to the shallowness of the epithelial layer. In situ labeling detected apoptotic cells in the spinous and granular cell layers of cholesteatoma tissue sections and similar findings in the normal external skin specimens. Western blot analysis confirmed that the expression of involucrin protein was the same in both wild-type SCC-25 cells and the SCC-25/bcl-xL transfectants.

Conclusions: In both the cholesteatoma epithelium and the normal external ear canal epithelium, differentiation and apoptosis begin when the epithelial cells separate from the basal cells. The mechanisms behind these changes, at least in apoptosis, appear to be controlled by the expression of the Bcl-xL protein.


The histopathological features of middle ear cholesteatoma are characterized by invasion of the tympanic cavity and mastoid cavity by keratinized squamous epithelium and by reversion of the auditory ossicles and surrounding tissues, which occurs below the invading epithelial layer. Numerous reports have dealt with the mechanism of the proliferation of cholesteatoma epithelial cells, and the cholesteatoma epithelium has generally been thought to have hyperproliferative properties. Previous studies have shown that the proliferative ability of the cholesteatoma epithelium is regulated by the status of inflammation below the epithelium. That is, when severe inflammation is present, the subepithelial inflammatory cells and the epithelial cells themselves produce various cytokines that accelerate the proliferation of the epithelial cells. Although the cholesteatoma epithelial cells undergo normal cell death, it has been demonstrated that proliferation of the epithelium is unlike the uncontrolled spread of cancer cells. Understanding of the mechanisms involved in the differentiation of the cholesteatoma epithelium is still poor, however. To date, various theories have been proposed, including that cholesteatoma epithelium is similar to normal skin epithelium in the pattern of expression of various cytokines and that it differs from such diseases as psoriasis and photokeratosis. Conversely, others think that the expression of CK16 is accelerated in cholesteatoma epithelium and that the epithelium has hyperproliferative properties. There has been almost no discussion of the degree of differentiation of the epithelium, however. Furthermore, at present, the mechanisms involved in the differentiation of epithelial cells and cell death are not understood. We think that greater comprehension of the mechanisms controlling the proliferation, differentiation, and apoptosis of the cholesteatoma epithelium is important.
MATERIALS AND METHODS

TISSUES

Tissue specimens were obtained from 10 patients with pars flacida–type middle ear cholesteatoma who underwent middle ear surgery at Jikei University School of Medicine, Tokyo, Japan, from November 1, 1997, through September 30, 1998. The patients (6 men and 4 women) ranged in age from 28 to 65 years (mean, 52 years). Specimens of normal external ear canal skin were obtained from the same 10 patients. The excised specimens were immersed in 10% buffered formalin, sliced into 3- to 4-µm-thick sections, and mounted on polylysine-coated slides.

IMMUNOHISTOCHEMICAL STAINING

Slides were deparaffinized and dehydrated in graded alcohol. Nonspecific binding was blocked with 10% normal horse serum and 1% bovine albumin for 20 minutes. After brief rinsing, the sections were immunoreacted with anti-Bcl-xL (Transduction Laboratories, Lexington, Ky) or anti-involucrin monoclonal antibody (Biomedical Technologies, Inc, Stoughton, Mass) at room temperature for 1 hour. The sections were washed in phosphate-buffered saline, incubated for 1 hour with biotinylated antirabbit IgG (1:100), and then treated with avidin-biotin complex for 1 hour; 3′-3′-diaminobenzene (Vector Labs, Inc, Burlingame, Calif) was then applied as a chromogen. Sections were viewed after counterstaining with hematoxylin.

IN SITU LABELING OF APOPTOTIC CELLS

Apoptosis was detected by labeling the 3′-hydroxyl ends of DNA using digoxigenin incorporation by 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 proteinase K, 20 µg/mL, at room temperature for 15 minutes. Endogenous peroxidase activity was quenched with 3% hydrogen peroxidase in phosphate-buffered saline. After digoxigenin-nucleotide was added catalytically, detection with antidigoxigenin peroxidase was performed using 3′-3′-diaminobenzidine for 6 minutes. The tissue sections were viewed after counterstaining with 0.5% methyl green.

RESULTS

A human tongue squamous carcinoma cell line, SCC-25 (American Type Cell Collection, Rockville, Md), was grown as monolayers in a Dulbecco modified–Eagle minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum; penicillin, 100 U/mL; streptomycin, 100 µg/mL; levoglutamide, 2 mmol/L, and hydrocortisone, 0.4-µg/mL. The SCC-25 cells were transfected with either the pSFFV-neo plasmid (SCC-25/neo) or pSFFV-neo containing bcl-XL (SCC-25/bcl-XL), as previously described.16 Transfectants were selected in the presence of neomycin sulfate, 400 µg/mL.

IMMUNOBLOT ANALYSIS

Cell lysates were prepared as described.15 Proteins were subjected to electrophoresis in a solution of sodium dodecyl sulfate and 10% polyacrylamide gel and then transferred to nitrocellulose membranes. The membranes were blocked with 5% dried milk, 0.1% polysorbate 20, and phosphate-buffered saline and then incubated with anti-involucrin monoclonal antibody (YLEM, Rome, Italy). After they were washed with phosphate-buffered saline and polysorbate, the membranes were incubated with horseradish peroxidase and conjugated antirabbit or antimouse IgG (Amersham Life Science). Antibody complexes were viewed by chemiluminescence (Amersham Life Science).

The exposed film was scanned into a Macintosh computer by an image scanner, and the intensity of the individual bands was quantified using commercial software (Image 1.37; Division of Computer Research and Technology, National Institutes of Health, Bethesda, Md).

Apoptosis is a fundamental part of normal development. The Bcl-2 protein—the product of the bcl-2 proto-oncogene—is a known inhibitor of apoptosis,13 and it is closely involved in terminal differentiation and apoptosis of the epithelium.19 Bcl-XL is a bcl-2–related gene, and—like Bcl-2 protein—Bcl-XL protein inhibits apoptosis of tumor cells due to various stimuli, such as DNA damage.15,16

We investigated the role of the Bcl-XL protein in the differentiation and apoptosis of cholesteatoma epithelium. We approached this by determining the localizations of expression of Bcl-XL protein and involucrin and the presence of apoptotic cells in tissue slices of middle ear cholesteatoma epithelium compared with the findings for normal external ear canal epithelium. Our results indicate that cholesteatoma epithelium shows the same patterns of differentiation and apoptosis as normal external ear canal epithelium and that the mechanisms behind these changes may be regulated by the expression of Bcl-XL protein.

Immunohistochemical analysis indicated that in all specimens of cholesteatoma and normal external ear canal skin, Bcl-XL was expressed in almost all cells of the basal cell layer and the lower region of the spinous cell layer (Figure 1). Involucrin was expressed in many cells of the upper region of the spinous cell layer and of the granular cell layer in specimens of both cholesteatoma and normal external ear canal (Figure 2). That is, Bcl-XL protein was observed in the region of the basal cell layer, but its expression decreased progressively in the superficial layers. Conversely, involucrin was expressed in the superficial layers. There was no difference between the normal external ear skin specimens and the cholesteatoma tissues in the expression of Bcl-XL protein or involucrin.

Next, we investigated whether the programmed cell death that occurs in conjunction with terminal differen-
tiation of epithelial cells differed between the normal external ear skin specimens and the cholesteatoma tissues. In situ labeling detected several apoptotic cells in the spinous and granular cell layers of cholesteatoma tissue sections. No apoptotic cells were observed in the basal cell layer (Figure 3). The pattern and kinetics of apoptotic cells in the control specimens were similar (Figure 3, bottom). By counting the live and dead cells, we quantitatively analyzed apoptotic cell death in at least 3 different areas of the sections. In the cholesteatoma epithelium, 63.8% ± 6.5% (mean ± SD) of the cells showed apoptosis. Similar results (60.1% ± 9.6%) were observed in tissue sections of normal skin. There was no statistical difference in the apoptotic index between the control specimens and the cholesteatoma tissues—ie, the cholesteatoma epithelium showed the same mechanisms of the regulation of cell death that are found in normal epithelium.

To elucidate the role played by the Bcl-xL protein (which is known to suppress apoptosis) in the differentiation and cell death of the cholesteatoma epithelium, the bcl-xL gene was transfected into the SCC-25 cell line to obtain SCC-25/bcl-xL cells that overexpressed the Bcl-xL protein. Using these transfectants, we investigated the effect of Bcl-xL protein on the differentiation of epithelial cells. Western blot analysis confirmed that involucrin was expressed the same as in wild-type SCC-25 cells and in the SCC-25/bcl-xL transfectants (Figure 4). In earlier studies,15,16 it was demonstrated that Bcl-xL protein suppresses apoptosis induced by various stimuli. That is, the cell death was suppressed, but the production of involucrin, a marker of epithelial cell differentiation, was not suppressed by the overexpression of Bcl-xL protein in the transfectants.

**COMMENT**

Various cytokines and growth factors are involved in the proliferation and development of cholesteatoma epithelium.4,5,7-9,18 When severe inflammation is present, the subepithelial inflammatory cells and the epithelial cells themselves produce various cytokines that accelerate the proliferation of the epithelial cells and are also thought to simultaneously destroy the underlying bone.7-9,19-21 In addition, it was demonstrated in an earlier study18 that
the cholesteatoma epithelial cells undergo normal cell death and that proliferation of the epithelium is unlike the uncontrolled spread of cancer cells. Many aspects of the regulation of the mechanisms of proliferation, differentiation, and apoptosis of the cholesteatoma epithelium, however, are still unclear.

Within normal stratified squamous epithelium, cells proliferate in the basal layer. As these cells migrate upward, they undergo terminal differentiation. Many genes control the switch from proliferation to nonproliferation by coordinating the regulatory pathways involved in terminal differentiation and programmed cell death.22,23

In the skin, Bcl-2 has been found only in the germinative basal epidermal layer and may be important for the maintenance of the stem cell compartment and the prevention of apoptosis of the basal keratinocytes.24,25 The \( bcl-xL \) gene was cloned as a \( bcl-2 \)-related gene.26 In humans, there are 2 kinds of complementary DNA (cDNA) for the \( bcl-xL \) gene: \( bcl-xL \) cDNA encodes a long type of Bcl-XL protein that has 233 amino acid residues, whereas \( bcl-xS \) cDNA yields a short protein consisting of 170 amino acids. The Bcl-XL protein shows 85% homology with the Bcl-2 protein, and—like the Bcl-2 protein—it suppresses apoptosis; in fact, Bcl-XL protein’s activity in suppressing apoptosis is even stronger than the activity of Bcl-2 protein. Conversely, Bcl-XS protein promotes apoptosis. The effects of Bcl-XL protein on the differentiation of epithelial cells, however, remain poorly understood. Our present studies did not reveal any differences in the patterns of expression of Bcl-XL protein in the cholesteatoma epithelium compared with normal external ear skin specimens. In both of these tissues, this protein was expressed mainly in the basal cell layer, but it was not detected in the cells of the upper epidermal layers.

Involucrin is a cytoplasmic protein (120 kd), a precursor of the epidermal cornified envelope that becomes cross-linked during envelope assembly.27 Involucrin is expressed in a broad range of stratified squamous epithelium, including the cornea, which lacks a distinct cornified layer. This protein is a useful marker of terminal differentiation. In normal dermis, involucrin is expressed in the upper cornified layer. In pathological conditions, however—eg, in psoriasis and other benign epidermal hyperplasias—involucrin expression is found closer to the basal layer.28 The expression of involucrin in the cholesteatoma epithelium, compared with in normal skin, was reported29 to extend from a lower epidermal layer through the upper layer. Our present study found that, in the cholesteatoma epithelium and control specimens, cells showing involucrin were located mainly in the upper epidermal layers; there was no difference between the 2 types of specimens. Furthermore, apoptotic cells were commonly observed in the superficial epidermal layers, but not in the basal cells, and again, no difference was found between the cholesteatoma epithelium and normal external ear skin.

The following hypothesis can be constructed on the basis of these results. First, apoptosis of epithelial cells is suppressed in the vicinity of the basal cells that express Bcl-x protein, and differentiation of the epithelial cells is being suppressed at the same time. The expression of Bcl-XL protein away from the basal cell layer, however, decreases, which results in down-regulation. This then leads to differentiation and cell death of the epithelial cells. It was recently reported30 that the Bcl-2 protein suppressed involucrin expression. With the objective of confirming this hypothesis, we created an SCC-25/bcl-xL transfectant that overexpressed the Bcl-XL protein and then compared the expression of involucrin—a known marker of cell differentiation—in these cells and in the wild-type SCC-25 cells.
We showed that the expression of the involucrin marker is not suppressed by overexpression of the Bcl-xL protein (ie, as in the SCC-25/bcl-xL transfectant cells).

As described earlier, we surmise that the processes of differentiation and cell death in the cholesteatoma epithelium are initiated when the epithelial cells separate from the basal cell layer. Furthermore, the mechanism underlying these processes is probably due to a progressive decrease in the expression of the Bcl-xL protein, at least in the cell death. These mechanisms in the cholesteatoma epithelium have been conserved, being the same as those in normal epithelium. On the basis of these mechanisms, we conclude that the cholesteatoma epithelium differs from other malignant epithelial tumors in that it does not cause uncontrolled proliferation of the cells.

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