Expression Patterns of Cytokeratins in Cholesteatomas: Evidence of Increased Migration and Proliferation

Aural cholesteatoma is characterized by invading squamous epithelia with altered growth properties. Cytokeratin (CK) expression is affected in epidermal proliferative diseases and represents the alterations of keratinocyte proliferation, differentiation, and migration. In the present study, the intensity of CK immuno-expression was determined, using densitometry at various sites in experimental cholesteatoma in order to characterize changes of keratinocytes. With cholesteatoma formation, CK4, a marker for non-keratinizing epithelia, increased in the suprabasal layers of the annular external auditory canal (EAC) and at the pars tensa indicating an altered differentiation and migration of keratinocytes. CK5/6, a marker of keratinizing squamous epithelium, increased only at the pars tensa of the tympanic membrane, indicating basal keratinocyte hyperplasia. CK1/10 increased in the suprabasal layer at the annular EAC, and at the peripheral pars tensa, indicating increased terminal differentiation of keratinocytes. CK13/16, markers of differentiation and hyperproliferation, increased in suprabasal layer of the EAC, and at the peripheral pars tensa. However, it decreased in the basal layer of the EAC, indicating hyperproliferation and migration of keratinocytes. The findings of this study support the basal cell hyperplasia hypotheses for the pathogenesis of aural cholesteatoma, with regard to hyperproliferation, migration, and an altered differentiation of keratinocytes.

Key Words : *Cholesteatoma; Models, Animal; Keratin; Keratinocytes; Densitometry*

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INTRODUCTION

It remains unclear whether cholesteatomatous epithelium is normal tissue merely in the wrong place or pathologic tissue with qualitative changes. Cytokeratins (CK) are known as the intermediate filament proteins of epithelial origin, and they comprise a family of at least 19 polypeptides. Each pair of acidic and basic CK is assembled into a 10-nm filament. The pattern of CK expression is affected only, if at all, by neoplasia (1) and some proliferative epidermal diseases including psoriasis, aural cholesteatoma, as well as other epithelial disorders (2-10).

Mongolian gerbils have a propensity for the development of aural cholesteatomas. Cholesteatomas were not only found to develop spontaneously with aging, but experimental cholesteatomas can also be induced by external auditory canal ligation (ECL) (11, 12). Induced cholesteatomas progressively enlarge to an advanced stage of cholesteatoma over time. Using this animal model, CK expression pattern can be investigated in various clinical stages of cholesteatoma. As patterns of CK expression indicate the state of keratinocyte proliferation, migration, and differentiation, this study might shed some insight into the pathogenesis of cholesteatoma.

In the present study, we investigated on the CK immuno-expression at various epithelial sites, i.e., the external auditory meatus, the tympanic membrane, and the middle ear, in various stages of gerbilline cholesteatomas of ECL model.

MATERIALS AND METHODS

Ligation of the external auditory canal

Twenty-eight normal mongolian gerbils (60-90 days old), *Meriones unguiculatus*, underwent the procedures and postoperative care. The procedures were performed in accordance with the PHS policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Care and the Animal Welfare Act (7 U.S.C. et seq); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Davis. Each animal was administered an intraperitoneal injection of ketamine HCl (Ketaset®, 50 μg/g)/xylazine hydrochloride (Rompun®, 12.5 μg/g) mixture for anesthesia. Under the operating microscope, the external auditory canal (EAC) and the tympanic membrane

(TM) were examined to exclude spontaneous cholesteatoma formation prior to surgery. An induction method of canal type cholesteatoma was followed as described in previous studies (11, 12). Ligation of the EAC was performed on the right ear, and the left ear was assigned as a control. Following surgery, the TM and the EAC of the left side were regularly examined to exclude spontaneous cholesteatoma formation.

Histopathologic preparation

Twenty-eight temporal bone specimen (56 external/middle ears) were processed for histopathologic examination. They were divided into seven groups by the time intervals following the surgery: 3 days, 1, 2, 4, 8, 12 weeks, and 1 yr. The animals were euthanized and transcardially perfused with 10% formalin solution in phosphate buffered saline. And then, the bullae were removed en bloc, immersed in the same perfusion fixative for 48 hr, and decalcified in 1.35 N hydrochloric acid with 0.003 M chelating agent (Cal-X®, Fisher Scientific Products) for 24 hr. Following decalcification, specimens were dehydrated, embedded in paraffin, and sectioned to 8 μm in thickness, along the superior/inferior axis through the EAC and the pars tensa, pars flaccida of the TM. En bloc slide specimens were collected and dried on poly-L-lysine coated slides.

Immunohistochemical staining

Sections were deparaffinized with xylene and rehydrated by serial alcohol solutions. The samples were then processed by the avidin-biotin-immunoperoxidase complex method. Antibodies were visualized by 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.3% hydrogen peroxide. The following antisera were used to identify CK changes: anti-pan CK (mixture of monoclonal antibodies to CK 1, 4, 5, 6, 8, 10, 13, 18, and 19, Sigma Co., St. Louis, MO, U.S.A.), K 8.60 (monoclonal antibody to CK10, 11, and weakly CK 1, Sigma Co.), D5/16 (monoclonal antibody to CK5 and 6, Boeringer-Mannheim, Germany), 6B10 (monoclonal antibody to CK 4, ICN, Costa Mesa, CA, U.S.A.), and K 8.12 (monoclonal antibody to CK 13 and 16, Sigma Co.). Negative controls were prepared by substituting the primary antibodies with normal sera. Since specific antibodies to gerbilline cytokeratins are not available, the antibodies used were commercially available bovine and human antibodies: (anti-CK1/10 and anti-CK 13/16 to bovine immunogens, anti-CK4 and anti-CK5/6 to human immunogens).

Densitometry

From each ear, data were collected from ten epithelial sites: cartilaginous, bony and annulus part of the upper and lower EAC (6 sites), pars flaccida, central and peripheral parts of pars tensa (3 sites) of the TM, and the middle ear (1 site). In the cartilaginous part of the EAC, data were collected from three separate layers of the epithelia: upper and lower suprabasal, and basal. In the bony and annular part of the EAC, and the pars flaccida of the TM, data were from two separate layers: suprabasal and basal. The intensity of immunostainings was measured quantitatively by video imaging densitometry. All videomicrographs (Olympus®) IMT-2, Burlingam, CA; mti® TV 70 series camera, Michigan City, IN, U.S.A.) were recorded directly from tissue sections to an optical disk (Panasonic®), TQ-2028F, Secacus, NJ, U.S.A.). Recorded images were played back and captured by Targa® M8 video-board (Truevision Inc, Indianapolis, IN, U.S.A.). The intensities were measured using Java® histomorphometric software (Jandel Scientific, Corte Madera, CA, U.S.A.). A control image of the optical path was subtracted from each image just before measurements were done. Five successive measurements were repeated at each site. Data were then transferred into a spreadsheet (QuatroPro®), and statistically analysed by ANOVA and Fisher's LSD test, using NCSS® statistical analysis program (version 5.0).

RESULTS

Experimental cholesteatomas were divided into five groups according to the stage of cholesteatoma (11, 12); control (n= 28), stage I (n=7), stage II (n=16), stage III (n=3), and stage IV (n=2). The results of CK expression throughout the study are summarized in Table 1.

Pan-Cytokeratin

Pan-CK expression was normally seen in the whole layers of squamous epithelia of the EAC and the TM, although the intensity of expression was higher in the suprabasal layer than in the basal layer. Following cholesteatoma formation, the expression patterns were changed: (1) the expression of the suprabasal layers in the lower cartilaginous EAC decreased in stage II and the expression of the bony EAC decreased in stages I cholesteatoma; (2) the expression in the pars tensa of the TM increased in stages II and III cholesteatoma (Fig. 1).

Cytokeratin 4

CK4 was occasionally expressed in the suprabasal epithelial layers of normal specimen. The expression in the lower annulus of the EAC and the pars tensa of the TM increased in stages II and III cholesteatoma (Fig. 2).

Cytokeratin 5/6

CK5/6 was normally expressed in the basal layers of the EAC and pars flaccida of the TM. This pattern was not changed but the intensity of expression in the peripheral part of pars tensa increased in stages I and II cholesteatoma (Fig. 3, 5).
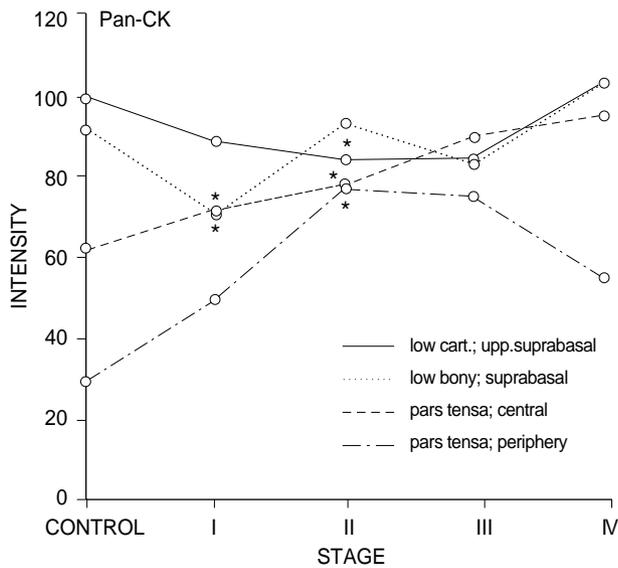
Fig. 1. The intensity scores of cytokeratin immuno-staining were plotted over the stage of cholesteatoma. Mean data for each cytokeratin and each site were presented. Asterisks represent the intensity scores with statistically significant differences from those of the control group (*p<0.05, ANOVA and Fisher's LSD). The staining intensity of pan CK is decreasing in the cartilaginous EAC of stage II and the bony EAC of stage I, but is increasing in the pars tensa of stage II and III cholesteatoma.
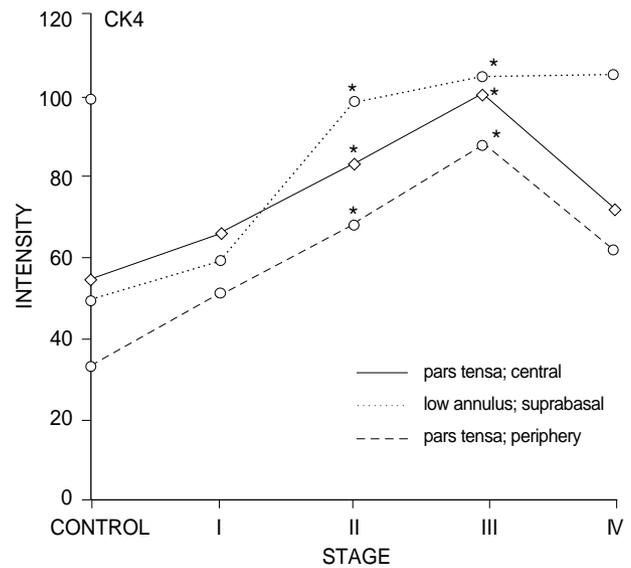

Fig. 2. The intensity scores of cytokeratin immuno-staining were plotted over the stage of cholesteatoma (*p<0.05). CK4 is increasing in the annular EAC, and the pars tensa of stage II and III.
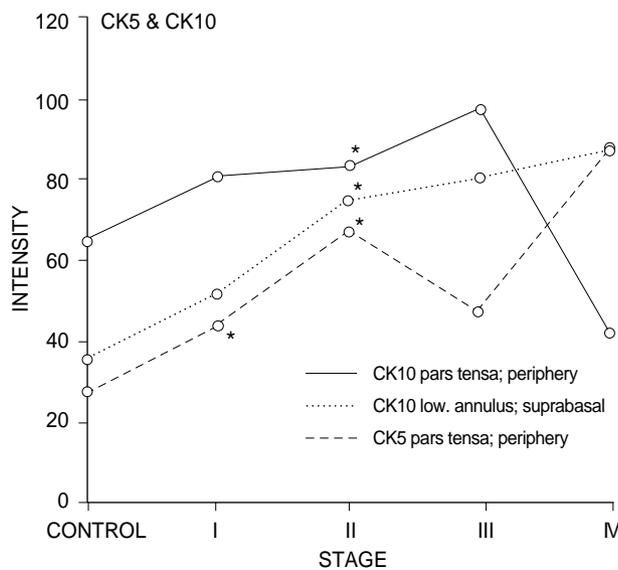

Fig. 3. The intensity scores of cytokeratin immuno-staining were plotted over the stage of cholesteatoma (*p<0.05). CK5 is increasing only in the pars tensa of stage I and II, while CK10 is increasing in the suprabasal layer of the annulus and in the pars tensa of stage II.
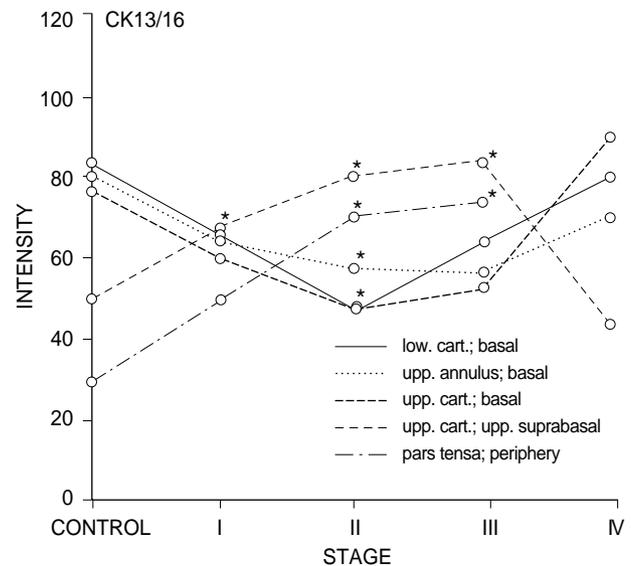

Fig. 4. The intensity scores of cytokeratin immuno-staining were plotted over the stage of cholesteatoma (*p<0.05). CK13/16 is increasing in the suprabasal layer of the EAC of stage I through III, and in the pars tensa of stage II and III. In contrast, it is decreasing in the basal layer of the EAC of stage II and in the annulus of stage III.

sity in the suprabasal layers of the lower annulus, and in the peripheral part of pars tensa increased in stage II cholesteatoma (Fig. 3).

Cytokeratin 1/10

CK1/10 was normally localized in the suprabasal epithelial layers of the EAC and the pars flaccida of the TM. The inten-

Cytokeratin 13/16

In normal specimens, CK13/16 was expressed in the basal epithelial layer of the EAC (Fig. 6A). However, in stages I
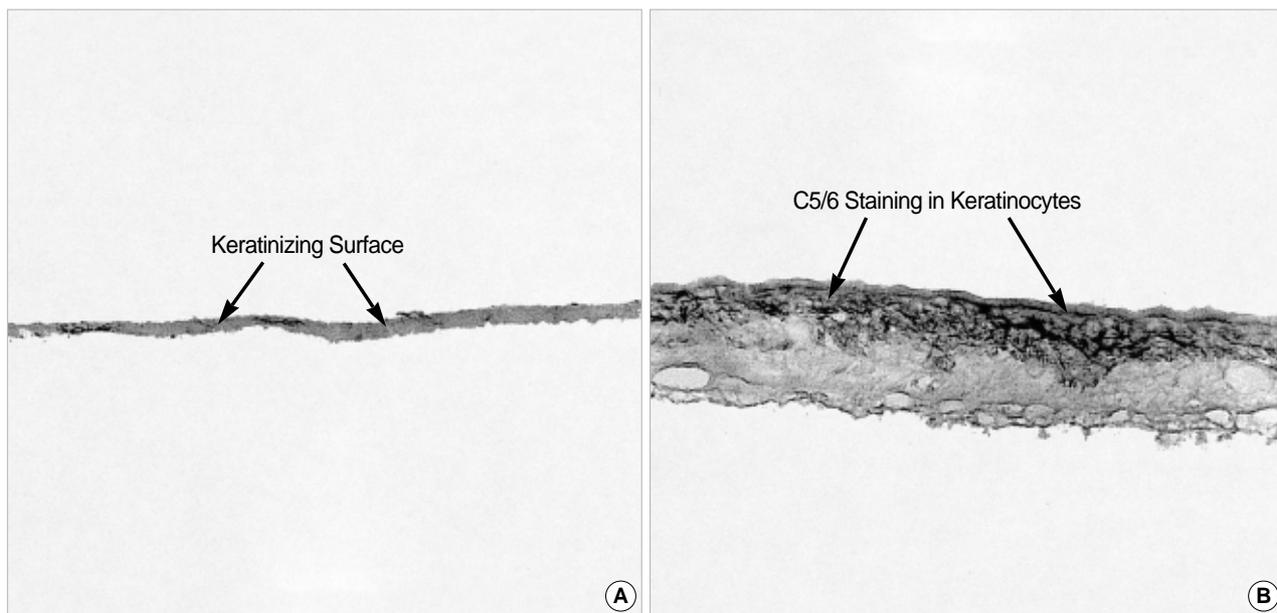
Fig. 5. Representative figures of cytokeratin immuno-staining in control and cholesteatoma tissues. ×200. **A**. CK5/6 is not expressed in the pars tensa of normal tympanic membrane. **B**. CK5/6 is intensely stained in the same site in cholesteatoma.
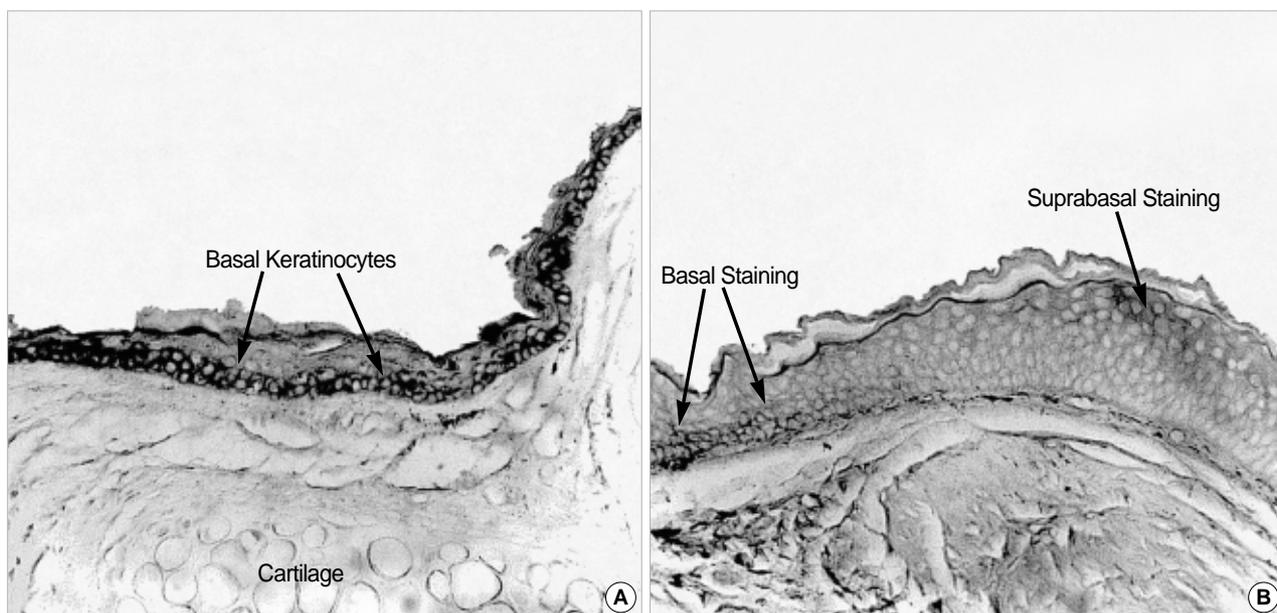

Fig. 6. A. CK13/16 is stained in basal layer of the cartilaginous canal in normal specimen. **B**. Transition of CK13/16 expression from basal to suprabasal layer is noticed in the same cholesteatoma tissue. Basal on the left, and suprabasal on the right of the photograph.

through III cholesteatoma, it was seen in the suprabasal layer of the EAC. A transition of expression from basal to suprabasal layer was occasionally seen in cholesteatoma epithelia (Fig. 6B). Interestingly, the expression pattern seemed to be back to normal in stage IV cholesteatoma. In contrast, the intensity of expression in the peripheral pars tensa of the TM increased throughout the stages (Fig. 4).

DISCUSSION

The patterns of cytokeratin (CK) expression correlate well with the state of keratinocyte proliferation, migration, and differentiation. These patterns are known to be affected during the formation of aural cholesteatoma. In the present study, gerbilline cholesteatomas of canal ligation (ECL) type were classified by the extent, i.e., the stage of cholesteatoma (11,

Table 1. The change of cytokeratin expression at various epithelial sites according to the stage of cholesteatoma

	Site		Basal	Lower suprabasal	Upper suprabasal
CONTROL (N=29)	External ear canal	upp. cart.	13		13
		upp. bony			
		upp. annl.	13		
		low. cart.	13		pan
		low. bony			pan
		low. annl.			4,10
	TM	p. flaccida			
		p. tensa ctr	Pan, 4		
		P. tensa peri	pan, 4, 5, 10, 13		
	Middle ear mucosa				
STAGE I (N=7)	External ear canal	upp. cart.	-	-	13 ↑
		upp. bony	-	-	-
		upp. annl.	-	-	-
		low. cart.	-	-	-
		low. bony	-	-	pan ↓
		low. annl.	-	-	-
	TM	p. flaccida	-	-	-
		p. tensa ctr	-	-	-
		P. tensa peri	5 ↑	-	-
	Middle ear mucosa		-	-	-
STAGE II (N=18)	External ear canal	upp. cart.	13 ↓	-	13 ↑
		upp. bony	-	-	-
		upp. annl.	13 ↓	-	-
		low. cart.	13 ↓	-	pan ↓
		low. bony	-	-	-
		low. annl.	-	-	4 ↑, 10 ↑
	TM	p. flaccida	-	-	-
		p. tensa ctr	pan ↑, 4 ↑	-	-
		P. tensa peri	pan ↑, 4 ↑, 5 ↑, 10 ↑, 13 ↑	-	-
	Middle ear mucosa		-	-	-
STAGE III (N=2)	External ear canal	upp. cart.	-	-	13 ↑
		upp. bony	-	-	-
		upp. annl.	-	-	-
		low. cart.	-	-	-
		low. bony	-	-	-
		low. annl.	-	-	4 ↑
	TM	p. flaccida	-	-	-
		p. tensa ctr	pan ↑, 4 ↑	-	-
		P. tensa peri	4 ↑, 10 ↑, 13 ↑	-	-
	Middle ear mucosa		-	-	-
STAGE IV (N=2)	External ear canal	upp. cart.	-	-	-
		upp. bony	-	-	-
		upp. annl.	-	-	-
		low. cart.	-	-	-
		low. bony	-	-	-
		low. annl.	-	-	-
	TM	p. flaccida	-	-	-
		p. tensa ctr	-	-	-
		P. tensa peri	5 ↑, 10 ↑	-	-
	Middle ear mucosa		-	-	-

Up/down arrows indicate the significant increase/decrease; "-" marks indicate no change of cytokeratin expression from the control group ($p<0.05$, ANOVA and Fisher's LSD). upp.: upper; low.: lower; cart.: cartilaginous; annl:annulus; p.: pars; ctr: center; peri: periphery; pan: panCK; 4: CK4; 5: CK 5/6; 10: CK1/10; 13: CK13/16.

12). CK4 was considered to be a marker of non-keratinizing squamous epithelium, CK5/6 a marker of basal keratinocytes and hyperproliferation, CK1/10 a marker of differentiation or keratinization of squamous epithelium, and CK13/16 and

Table 2. Epithelial characteristics associated with tested cytokeratins

Cytokeratin	Associated with
PanCK	All epithelia
CK1/10	Cornification and terminal differentiation
CK4	Non-cornyfying epithelium
CK5/6	Hyperproliferation of basal keratinocytes
CK13/16	Hyperproliferation and terminal differentiation

CK5/6 markers of basal keratinocytes and hyperproliferation (Table 2).

Pan-CK polyclonal antibody is normally expressed in all epithelial tissues. In the present study, the staining intensity of pan-CK was weaker in the basal layer of the epithelium in the EAC in the normal specimen than in the suprabasal layer. This phenomenon has been partially explained by some investigators advocating that some antigenic determinants are masked in situ in certain cell layers, especially if polyclonal antibodies are used (13). With cholesteatoma formation, pan-CK expression appeared to be less intense in the suprabasal layers of the EAC, but it became more intense in the pars tensa of the TM. This finding suggested that different patterns of CK expression might exist separately in the EAC and the TM. Hence, immunohistochemical data collected from a single part of disease or in an uncertain time frame of the disease should be cautiously interpreted because they might be misleading. It is still unclear whether the different patterns of expression represent actually qualitative changes or simply uneven distributions of CK.

Previously, we found that epithelial hyperplasia in experimental cholesteatoma was more prominent in the early stage than in the advanced stage (11). These findings suggested that the epithelial expression of CK in cholesteatoma might be accordingly different in various clinical stages of the disease. The results of the present study also show that the expression patterns of each CK differed with respect to location and severity of the disease and support the concept that some keratinocytes in cholesteatomas show an increased proliferation.

CK 5 and 6 are found in epidermis and hair follicles and are closely related to each other as judged from peptide maps (1); these CK are found in proliferating epidermis and hair follicles. The results of this study show that the expression of CK 5/6 increased in the pars tensa of cholesteatoma, compared to controls. Increased CK5/6 expression in the pars tensa of developing cholesteatomas might be an indication of basal cell hyperproliferation, which supports the basal cell hyperplasia hypothesis for cholesteatoma pathogenesis.

Both CK6 and 16 may represent markers for hyperproliferative keratinocytes in general (4, 7). In various epidermal diseases, there is a reciprocal expression of the keratin markers for hyperproliferation and keratinization, supporting the mutual exclusiveness of the cellular events (9, 10). At the margin of the active psoriatic lesions, the suprabasal K1.12

(CK 13, 16) binding was the earliest change found in the epidermis (14). In the present study, an increase of CK13/16 expression was also found in the pars tensa of the TM, suggesting that a hyperproliferative process might be present at this epithelial site during cholesteatoma formation. Therefore the increased expression of CK5/6 and CK13/16 in the pars tensa of cholesteatomas suggests that hyperproliferation of keratinocytes within the cholesteatoma may lead to further advancement of the cholesteatoma and adds support to the basal cell hyperplasia theories of cholesteatoma formation.

Although CK4 and CK13 were usually expressed in the non-keratinizing epithelia in oral mucosa (9, 10, 15, 16), CK4 and13 have been demonstrated in some matrices of cholesteatoma indicating disturbances in the terminal differentiation. In the present study, CK4 expression in the annular part of the EAC and the pars tensa of the TM revealed a significant increase. This result indicated that CK4 expression, as a marker of disturbances in keratinocyte differentiation, increased in the deepest meatus and the TM. This finding suggests that qualitative changes in keratinocytes of cholesteatoma were generated at the areas of most rapidly expanding part in cholesteatoma. Together with the findings of keratinocyte proliferation described above, this finding might also support epidermal migration theory of cholesteatoma pathogenesis.

CK5 and CK14 are synthesized in the basal epithelial cells, and are used as markers of keratinizing squamous epithelium (1, 2, 3, 18). The presence of this pair of CK was detected in human cholesteatoma tissue by using SDS-PAGE (6). The epidermis of cartilaginous part showed a normal skin type differentiation, characterized by expression of CK5/14 in the basal layer, and CK10 in the suprabasal layer. The existence of this paired CK in the specimens of human middle ear cholesteatoma strongly favors the migration theory in the genesis of cholesteatoma, as Broekaert et al. mentioned (3).

Some authors have designated the cholesteatoma tissue as type A and type B according to the presence of CK13 and CK16. The suprabasal layer was selectively stained in type A, whereas the basal layer was stained in type B. They suggested that type A is an extension of meatal skin, which has retracted to the middle ear cavity, and type B is the cholesteatoma tissue itself. They further hypothesized that cholesteatoma may be composed of epithelium that has transformed from type A (near the external canal) to type B during cholesteatoma growth (7). In the present study, those CK were normally expressed in the basal epithelial layer; in contrast, they were also expressed in the suprabasal layer of the cholesteatomatous epithelium. Surprisingly, there was an area with a transitional expression pattern from the basal to suprabasal layer in some specimens. Some authors have argued that the deeper part of the EAC might be the areas of proliferation from studies of CK6 and 16 expressions. However, others believed that the presence of CK6 and 16 in the deep meatal skin would be related to a specific migration process (14). In the present

study, the increased CK13/16 expression was found in the pars tensa of the TM and the suprabasal layer of the deeper EAC. This result may suggest that proliferation of keratinocytes result in an increased migration at the most rapidly expanding epithelial site of cholesteatoma.

Cytokeratin expression has been noted to correspond to the extent of differentiation within keratinocytes. More highly differentiated keratinocytes express CK1/CK10, whereas the non-keratinizing epithelia in oral mucosa express CK4/CK13. In the present study, we observed an increase in both CK4 and CK10 with advancing cholesteatomas. CK4 expression was seen to increase in the suprabasal layer of the epithelium of the EAC as the cholesteatomas advanced in size. Other investigators observed an increased CK4/13 expression in some matrices of cholesteatoma indicating disturbances in the terminal differentiation (14, 17, 18).

CK1 and CK10 are synthesized in the suprabasal layer and are recognized as markers of increased differentiation or cornification of the squamous epithelium. In the present study, we observed an increased CK10 expression in the suprabasal layer near the annulus and the peripheral pars tensa in cholesteatomas. Some authors have demonstrated a decrease of the CK10 expression in cholesteatoma and proposed that the terminal keratinization might have been inhibited by the diseased state (3, 8, 19). These results seem somewhat contradictory, but it may be due to the different methods of sampling and analysis. Previous studies (3, 6, 8, 19) did not describe which portion of cholesteatoma had been collected, nor how advanced their cholesteatomas were. On the other hand, in the present study, immuno-expression data were collected from defined areas of the cholesteatoma in different stages of the disease. Our results suggest that CK10 expression may be affected by the extent of cholesteatoma and the severity of disease. Cytokeratin expression in these experimental cholesteatomas appear to indicate that at certain sites within a cholesteatoma some keratinocytes may differentiate into highly keratinizing cells (CK10), while others appear to resemble less differentiated keratinocytes (CK4), possibly indicating some defects in the control of keratinocyte differentiation. Finally, the interpretation of these data must be tempered by the possibility that gerbil cytokeratin may not share the exact antigenic characteristics of murine and human keratin and the antibodies used in this study.

In summary, the findings of this study support the concept that the normal keratinizing epithelium of the TM undergoes changes, as it forms a cholesteatoma in the external canal and middle ear. Our finding of increased CK5/6 and CK13/16 expression provides further evidence that the epithelium within cholesteatoma shows increased proliferation of keratinocytes, especially of the pars tensa near the malleus. Another evidence for hyperproliferation is based on increased rates of cell division (20) and increased expression of proliferating cell nuclear antigen (PCNA) (21). This proliferation also appears to be associated with increased keratinocyte migration. The increased CK4 expression in the deepest meatus near the TM and the transition of CK13/16 expression from basal to suprabasal layer suggested that the epithelial migration might be increased from lateral to medial portion within the auditory canal. The result of increased CK4 and CK1/10 expression might suggest an alteration of terminal differentiation in cholesteatoma pathogenesis.

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