IMMUNOHISTOCHEMICAL ANALYSIS OF THE CYTOKERATIN EXPRESSION IN MIDDLE EAR CHOLESTEATOMA AND RELATED EPITHELIAL TISSUES

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Immunohistochemical investigations were carried out to determine the pattern of cytokeratin (CK) expression in middle ear cholesteatoma and related epithelia. Using monoclonal antibodies specific for CK chains and the indirect immunoperoxidase technique, we examined 10 CK polypeptides for expression. The external stratified squamous epithelium of the tympanic membrane generally expressed Cks 5, 10, and 14. In addition, basal keratinocytes in the annular region of the pars tensa expressed CK 19 (a simple epithelium marker), while suprabasally the hyperproliferative marker CK 16 was expressed. These data reflect the unusual proliferative nature of this region. The unexpected appearance of CK 16 (known to have a limited distribution in healthy epidermis) clearly relates to its expression in the neighboring deep meatus. The medial simple epithelium of the eardrum revealed mucosal Cks 7, 8, 14, 18, and 19. Acquired cholesteatoma lesions, besides Cks 5, 10, and 14, consistently expressed CK 16 in suprabasal layers. These results constitute the first direct molecular evidence for the hyperproliferative nature of the cholesteatoma matrix. Overall, our CK data suggest that aural cholesteatoma lesions and epidermal tissue in this area are related. However, they do not explain the mechanism(s) by which the eardrum or mesal epithelia might invade the middle ear cavity. Congenital cholesteatomas expressed Cks 5, 10, 14, and 16 equally. These CK data do not support the idea of a metaplastic origin from middle ear mucosa; instead, they suggest activation of an ectodermal rest in the middle ear cavity.

KEY WORDS — cytokeratin, cholesteatoma, mesal epithelium, middle ear cholesteatoma, middle ear mucosa.

INTRODUCTION

In spite of numerous efforts to explain the presence of an expanding stratified squamous epithelium within the middle ear cavity, cholesteatoma pathogenesis is still a matter of debate, as was evident at the Second and Third International Conferences on Cholesteatoma and Mastoid Surgery. Our intention has been to try to fill the many gaps in the knowledge of cholesteatoma pathogenesis by a fundamental approach to the differentiation of the cholesteatoma matrix. We focused our investigations on markers of soft keratinization and utilized epidermal tissues as a reference. Previously, we surveyed the behavior of the nucleus, the cross-linking of structural polypeptides, and the role played by epidermal transglutaminase. These studies showed that cholesteatoma manifests a keratinization of the epidermal type, although the balance of early versus late stages of keratinization is altered in favor of the early ones. In addition, terminal keratinization is defective as far as cross-linking near the cellular membrane is concerned.

Recently, we also reported on the cytokeratin (CK) and vimentin expression in acquired middle ear cholesteatoma lesions. The CKs are prominent differentiation markers in epithelial cells, constituting in humans a complex family of 19 closely related polypeptides subject to differential (co)expression. The composition of the CK subsets depends on the epithelial type and location, the cell type, and the differentiation state reached in stratified epithelia. In pathologic conditions, the expression rules are equally valid. As applied to the cholesteatoma project, we determined that the CK profile in the matrix is of the epidermal type, although we found some expression of nonepidermal CKs. Probing both the early and late phases of differentiation, we again found certain alterations suggesting a hyperproliferative disease. In the meantime, this work has been largely confirmed by parallel studies.

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The primary purpose of the present study was to extend our observations to the outer epidermal layer and to the inner simple epithelium of the eardrum, in which we have systematically looked for differences in the CK expression between the various anatomic regions. These regions have a distinct ultrastructural morphology and present a different frequency of perforations, leading to various clinical forms of cholesteatoma, perhaps mediated by different etiologic factors. Second, we have been able to reconstruct the CK expression throughout the meatus, since skin autopsy specimens from precisely circumscribed sites were available. Third, the availability of new antibodies justified an additional screening of tissues for expression of some CK polypeptides that were beyond the scope of our previous investigations. Among these is CK 16, a major CK polypeptide in hyperproliferative conditions. Finally, for the first time we included a small group of primary or congenital cholesteatomas. The etiology of these lesions concentrates on the activation of an embryonic or ectodermal rest in the middle ear cleft. Those who reject a congenital origin refer to metaplasia of middle ear mucosa due to inflammation.

MATERIALS AND METHODS

Materials. Biopsy sampling from reference epidermal tissues, middle ear mucosa, and acquired cholesteatomas was exactly as described before. Full-thickness sheets of skin were dissected at regular distances from the bony and cartilaginous parts of the canal. Congenital cholesteatomas were screened with the utmost strictness and were obtained from children with a perfectly normal eardrum and without any previous otitis media or history of prior middle ear disease. All biopsy specimens stored in liquid nitrogen were processed for cryosectioning (5 μm) at the appropriate time.

Intact tympanic membranes (TMs) were dissected from cadavers (normal adults, both ears, 1 to 2 hours postmortem) by means of appropriate microsurgical instruments. A circumferential incision was made about 5 mm from the annulus, the annulus was lifted, and the whole TM and cuff of the adherent canal skin was removed with the malleus handle. Following rinsing with Hanks’ balanced salt solution, the whole TM and adjacent mental skin were prepared carefully under a binocular microscope. Subsequently, these specimens were stored temporarily in liquid nitrogen. Afterward, transverse cryosections (5 μm) were cut serially throughout the TM, providing at least 6 and at most 10 levels for close inspection.

Immunohistochemical Staining. For most parts of the study, frozen sections were processed for the indirect immunoperoxidase technique by using murine monoclonal antibodies. Air-dried cryosections (2 hours, 20°C), fixed in acetone for 10 minutes at 20°C, were rehydrated for 5 minutes in 10 mmol/L phosphate buffer, pH 7.2, containing 0.85% sodium chloride (PBS). Monoclonal antibodies were applied, either undiluted or diluted (1/5, 1/10, or 1/25 in PBS) for 30 minutes at 20°C in a moist chamber. Only LT18, 21D7, KKS.60, KKS.12, KKS.58, PAB601, and LL026 were applied as an undiluted culture supernatant or were diluted 1/2 in PBS. Tissue culture medium from nonproducing hybridomas was used as a negative control. Subsequently, slides were washed in PBS (jet clean followed by three washes, 10 minutes each, using a magnetic stirrer, the second wash containing in addition 0.1% Tween-20). Rabbit anti mouse immunoglobulin (Ig) conjugated to peroxidase (1/40, respectively, in 10% normal human serum in PBS) was used as a second antibody (30 minutes at 20°C). Probing for CK 5 by the rat monoclonal antibody 21D7 required the rabbit anti rat Ig peroxidase conjugate (1/40, respectively, in PBS enriched with 10% normal human serum) as a second antibody. The sections were washed for another 30 minutes as described above, except that 0.05 mol/L sodium acetate buffer, pH 4.85, was substituted for the third wash. 3-Amino-9-ethylcarbazole (0.2 mg/mL AB containing 0.01% hydrogen peroxide) was applied as the electron donor substrate for 5 to 10 minutes. Finally, sections were washed in running tap water, counterstained with Mayer’s hematoxylin (15 to 30 seconds), and mounted in Kaiser’s glycerin-gelatin (Merck No. 9242). A minor number of sections were stained by the indirect immunofluorescence method. In this method, 0.1% Tween-20 and 100 mmol/L potassium chloride in PBS was applied in the first wash, and rabbit anti mouse Ig conjugated to fluorescein or rhodamine (1/20 in 10% normal human serum in PBS) was used as the second antibody. Slides mounted in PBS-glycerin (1/1) were viewed with a Dialux 20 microscope (Leitz, Wetzlar, Germany) equipped with a Plomopak epifluorescence microscope (type 2.4) and the appropriate filter blocks for fluorescein (T2) and rhodamine (N2). All secondary antibody conjugates were purchased from Nordic Immunological Laboratories (Tilburg, the Netherlands).

Two panels of monoclonal antibodies were applied to the serial sections. The first panel (Table 110.15-24) was applied to only mental tissues, TM (5 samples), and congenital cholesteatomas (5 samples) to complete our earlier investigations. The second panel (Table 210.25-28) was used here for the first time on primary and acquired cholesteatomas (5 and 12
TABLE 1. FIRST MONOCLONAL ANTIBODY PANEL

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Corresponding Antigen(s)*</th>
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<tr>
<td>6B10</td>
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<tr>
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<td>CKs 5 and 8</td>
<td>16</td>
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<td>CK 7</td>
<td>17, 18</td>
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<tr>
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<td>19</td>
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<td>24</td>
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<td>18</td>
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<tr>
<td>RD301</td>
<td>Desmin</td>
<td>17</td>
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</table>

*Numbered according to catalog.  
†Broad-spectrum CKs including CK 5, and neurofilament polypeptides.  
‡Commercially available from Sanbio BV (Uden, the Netherlands).  
§Commercially available from Amersham (England).

samples, respectively) and all related and reference tissues.

Overall, 10 CK polypeptides were screened individually. To overcome false-negative results due to masking effects, repetitive screening of CKs 5, 8, 10, 13, 14, and 18 was performed by applying antibodies with different specificities. To detect potentially positive sites, nonspecific probes were used. Vimentin probes highlighted tissues of mesenchymal origin.

RESULTS

In this section, only positive data are described. Broad-spectrum (nonspecific) probes (clone 80, RCK103, and LP34) provided uniform staining of normal epidermis, lateral stratified squamous and medial simple epithelia of TM, middle ear mucosa, and cholesteatoma. The CK data are summarized in Table 3.

Metastatic Epidermis. Whether samples originated from deep in the canal (ie, near the annulus) or from more external sites, the presence of CKs 5, 10, and 14 was consistently demonstrated. The CK 5 and 14 staining was moderate in intensity and localized mainly in basal keratinocytes; it faded away in the suprabasal layers. In contrast, CK 10 staining was intense in the suprabasal layers. Kkx 8.60, specific for CKs 10 and 11, gave similar staining profiles. Unexpectedly, the metastatic epidermis exhibited weak to intense CK 16 staining, restricted to the suprabasal keratinocytes in TM-associated regions (Fig 1A).

TABLE 3. SURVEY OF IMMUNOHISTOCHEMICAL CYTOKERATIN DATA

<table>
<thead>
<tr>
<th>Meatal Epidermis</th>
<th>Typanic Membrane</th>
<th>Middle Ear Mucosa</th>
<th>Acquired Cholesteatoma</th>
<th>Congenital Matrix</th>
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<tr>
<td></td>
<td>Lateral Side</td>
<td>Medial Side</td>
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<tr>
<td>CK 4</td>
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<td>-</td>
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<td>CK 5</td>
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<tr>
<td>CK 16</td>
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<td>+</td>
<td>±</td>
</tr>
<tr>
<td>CK 18</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>CK 19</td>
<td>±f</td>
<td>±f</td>
<td>+</td>
<td>±</td>
</tr>
</tbody>
</table>

* Combined data from previous paper2 and present investigations.
† Expressed in pseudostratified epithelial foci.
‡ Focal staining, associated with terminal keratinization in certain cases.
§ Staining restricted to deep metatus.
An Extremely weak staining in central areas, markedly intensified in annular regions.
f Staining of basal keratinocytes in annular regions of lower part of tympanic membrane.
This unusual expression of CK 16 was observed in deep canal samples from cholesteatoma patients and from normal controls. In the other regions of the meatus, CK 16 staining was limited to the external root sheath of the hair follicles. Ks8.12 and Ks8.58, both specific for CKs 13 and 16, yielded a significant staining of basal keratinocytes. Sometimes this staining extended throughout the malpighian layer (Fig 1B). We also observed the presence of CK 19 in basal keratinocytes of annulus-associated regions opposite the pars tensa (Fig 1C).

A specific search for CKs 16 and 19 in the interfollicular epidermal regions (eg, breast, abdomen, shoulder, arm, and retroauricular area) yielded no positive staining, except for CK 19 in scanty Merkel’s cells. Thick epidermal tissues from pressure-sensitive sites (eg, thumb, sole, heel), however, consistently yielded weak to moderately intense CK 16 staining, visible suprabasally in the living layers.

Finally, vimentin reactivity was present exclusively in Langerhans’ cells, spread among keratinocytes.

Tympanic Membrane. The lateral stratified squamous epithelium clearly displayed a CK pattern characteristic of epidermal tissue. As a rule, staining profiles were similar throughout the pars flaccida, the pars tensa, and the annular regions. The CK 5 antibody 21D7 provided weak labeling of both living and dead layers. Alternatively, the RCK102 staining of CK 5 in the absence of CK 8 was maximally intense in basal keratinocytes, and minimal in final keratinization areas. Cytokeratin 10 was a prominent marker of all suprabasal stages, and Kk8.60 produced a similar staining profile. Cytokeratin 14 staining was weak and restricted to the deeper layers.

Throughout the TM, from the umbo to the annulus, we observed weak LL026 staining, suggesting a minor suprabasal expression of CK 16. Near the annulus, however, the staining increased, clearly indicating CK 16 in suprabasal keratinocytes (Fig 2A). Cytokeratin 19 was present in basal keratinocytes in the annular region bordering the pars tensa. Significant labeling produced by Ks8.12 and Ks8.58, indicative of CK 13 and/or CK 16, was observed throughout epithelial layers at all sites (Fig 2B). Finally, staining for vimentin revealed very few Langerhans’ cells, except near the annulus.

The epithelium lining the medial side (in central areas), and adjacent to the mucosal lining of the tympanic cavity, significantly expressed CKs 7, 8, (M20-positive although LE41-negative), 14 (LH8-positive, PAB601-nonreactive), 18, and 19. Rare pseudostatified columnar foci expressed CKs 4 and/or 13. RCK102 provided uniform staining, while Ks8.12 and Ks8.58 staining was restricted to columnar cells, leaving squamous cells unstained.
Fig 2. Immunohistochemical staining profiles of lateral stratified squamous epithelium of tympanic membrane. Tissue slices were routinely counterstained with Mayer’s hematoxylin. ct — connective tissue, bars — 30 μm. Other abbreviations as in Fig 1. A) Expression of CK 16 in suprabasal layers near annulus (LL026 staining). B) Significant Ks8.12 labeling throughout all epithelial layers.

Middle Ear Mucosa. The CK reactivity in middle ear mucosa, screened with the second antibody panel (Table 2), was identical to the reactivity of the medial simple columnar epithelium of the TM; ie, only M20, Ks8.12, and Ks8.58 provided substantial staining.

Acquired Middle Ear Cholesteatoma. Using the new antibody panel (Table 2), we reconfirmed the obligatory expression of CKs 5, 10, and 14 during matrix differentiation. The antibody 21D7 weakly stained matrix tissues in all cases. Some staining extended throughout the accumulated keratinous debris (11 of 12 cases). Cytokeratin 10 staining, as a rule, was more intense and confined to the suprabasal layers. However, only the suprabasal layers in advanced stages of differentiation were stained in 8 cases. In 4 other cases, the CK 10 staining was present from the first suprabasal layer onward. Ks8.60 generated similar staining profiles. Cytokeratin 14 again was the predominant marker of the lower matrix half. Progressive loss of CK 14 characterized terminal differentiation.

The hyperproliferative marker CK 16 was consis-

Fig 3. Immunohistochemical staining profiles of acquired middle ear cholesteatoma. Tissue slices were routinely counterstained with Mayer’s hematoxylin. pm — perimatrix, bars — 30 μm. Other abbreviations as in Fig 1. A) Prominent LL026 staining of suprabasal matrix keratinocytes, indicating expression of CK 16. B) Typical Ks8.12 labeling profile, ie, diffuse minimal staining of basal keratinocytes and moderate staining of suprabasal matrix cells, completed by maximal staining of keratinous debris.
tently identified in the suprabasal matrix cells. In general, well-developed matrix configurations acquired a stronger staining than thinly stretched matrix tissues (Fig 3A). The corresponding epitope largely appeared in the adjacent stratum corneum areas in 4 of 12 cases, but was not evident in the horny layers in 8 other cases. KS8.12 and KS8.58 both provided similar staining profiles, distinct from the L1.026 profile. In 6 of 12 cases basal keratinocytes showed diffuse minimal staining, while in 4 cases they revealed maximal labeling. Suprabasal cells retained a positive staining, which was most prominent in the keratinous debris (Fig 3B).

**Congenital Cholesteatoma.** Our preliminary data on five primary cholesteatomatous indicate the presence of Cks 5, 10, and 14 in an epidermal mode, and of CK 16 exclusively in suprabasal matrix cells. In three cases, the expression of the CK 16 epitope was occasionally present in the stratum corneum. The expression of certain nonepidermal Cks, sometimes noted in acquired cholesteatoma tissues,9 was not observed.

**DISCUSSION**

The present data permit discussion of the state of differentiation reached in middle ear cholesteatoma tissues.

Although the meatal epithelium provided a typical epidermal CK pattern, the deep meatus was unique. In addition to Cks 5, 10, and 14, expressed at the appropriate level of differentiation, we could see suprabasal CK 16 in healthy as well as in cholesteatoma tissue. Cytokeratin 16 has previously been identified as a molecular marker of hyperproliferative keratinocytes.11,29 It has been observed in only a limited number of healthy epidermal sites (ie, soles and outer root sheaths of hair follicles).10 We found CK 16 in other pressure-sensitive sites having a stiff, thickened epithelms (eg, heel and thumb). In contrast, all thin epithelial tissues, including those from the cartilaginous and lateral bony parts of the meatus, were nonreactive.

The unusual character of the boundary area of the meatus and the eardrum is further documented by the presence of CK 19, which is a major constituent of simple epithelia and to some extent of certain pseudostratified epithelia of internal organs.10,11 Cytokeratin 19 in epidermal basal keratinocytes has never been reported and is difficult to evaluate. However, by analogy with the staining pattern seen in other stratified squamous epithelia (eg, conjunctiva),30 CK 19 may reflect the presence of proliferating stem cells.

The lateral stratified squamous epithelium of the TM displayed an epidermal CK pattern in the pars flaccida, the pars tensa, and the annular region. A supplementary expression of CK 16 was noticed in suprabasal keratinocytes of annulus-associated regions. From there to the umbo, staining decreased. Cytokeratin 19 was invariably identified in basal keratinocytes near the inferior annular part of the TM. In summary, the edge of the TM and the deep meatus displayed identical CK profiles, indicating an obvious continuity in this transitional area.

Our CK data show unusual proliferative capacity around the annular region opposing the pars tensa. This is consistent with earlier reports of a specific area of 3H-thymidine uptake and subsequent spreading.21-35 Similar conclusions can be drawn from wound closure experiments35,36,37 and from studies on the course of fetal and postnatal growth of the eardrum and deep canal.38

The CK composition of the medial simple epithelium of the eardrum, whether composed of columnar or squamous cells, is very similar to that of the middle ear mucosa, as noted in our previous study.9 In this study, we have also collected convincing evidence that CK 8 belongs to the mucosal CK family. This finding is also supported by other current studies.12,13 Mucosal CK (in addition to CK 14) is composed primarily of typical simple epithelial Cks, ie, Cks 7, 8, 18, and 19, and conforms to rules of CK expression.11 As a consequence, the question of whether CK 5 is expressed in middle ear mucosa, which was indirectly postulated in the past,9 is now open for discussion. We obtained negative results with the 21D7 antibody, although CK 14, the natural coexpression partner of CK 5, is present. Finally, we observed CK 4, a marker of nonepidermal stratified epithelia,11 in mucosa in our earlier study,9 but actual data show CK 4 only in pseudostratified foci on the medial side of the TM.

Concerning primary and acquired cholesteatomas, we confirmed our previous conclusions that Cks 5, 10, and 14 are expressed in an epidermal mode.9 Additionally, CK 16 was observed suprabasally in matrix tissues. Since CK 16 is a marker for hyperproliferative keratinocytes in general,11,29 this observation provides direct biochemical evidence for the hyperproliferative nature of cholesteatoma. This hyperproliferative state, whether focal, local, or general, has been postulated before, but evidence was only circumstantial.9 Here again, the expression of the advanced phenotypic keratinization marker CK 10 was slightly delayed, underscoring the hyperproliferative characteristic. Expression of nonepidermal
CKs was not observed in our small group of congenital lesions, contrary to reported experience with acquired lesions.\(^{9,12,13}\) This difference suggests that congenital cheilitestomas might evolve, more strictly than acquired lesions, through the soft keratinization route. The Ks8.12 and Ks8.58 staining profiles, representing a common epitope of CKs 13 and 16, were wider than the CK 16 patterns shown by LL026, although CK 13 apparently was absent from the CK complement. According to the CK expression rules and the actual tissue staining distribution, we presume that CK 15, in addition to CKs 13 and 16, was recognized by both antibodies.\(^{27}\)

The functional significance of human CKs is conserved under pathologic conditions.\(^{11,19}\) Thus, CK typing has predictive value in identifying pathologic states. In acquired cheilitestoma, the striking similarity between the CK complement in the matrix and in the epidermal tissues (deep meatus and/or lateral cover of the TM) suggests a pathogenetic link. Our immunohistochemical investigations do not explain the mechanism(s) by which the eardrum or meatal epithelium might invade the middle ear cavity. The CK profiles obtained do not support the metaplastic origin of middle ear cheilitestoma, however. In the case of a stratified squamous metaplastic lesion, a unique combination of one or more of the major CKs from the recipient tissue and stratification-related CKs has been reported.\(^{40,41}\) When the metaplasia, in addition, exhibits phenotypic keratinization including cornification, expression of terminal epidermal keratinization markers is expected. Our study demonstrates that the cheilitestoma CK complement does not have this metaplastic characteristic. Although this does not disprove the metaplasia theory, neither does it provide support for it.

A metaplastic origin of congenital cheilitestomas, likewise, is unsupported by our data. Instead of metaplasia, our data support the activation and expansion into a lesion of an embryonic or ectodermal rest in the middle ear itself. The existence of these islands is discussed in detail elsewhere.\(^{42}\)

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