Cytokeratins in Induced Epidermoid Formations and Cholesteatoma Lesions

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The expression of cytokeratins varies with the type of epithelium, the state of differentiation, and pathological conditions. In this study, the differential expression of cytokeratins in external meatal skin and middle ear epithelium was used for a pathogenetic study of cholesteatoma lesions and infection-induced epidermoid formations in the middle ear of the rat. Immunocytochemistry generally revealed an epidermal-type cytokeratin profile in the cholesteatoma matrix, except for the focal expression of nonepidermal cytokeratins at the invasion front. Comparable observations were made in the middle ear of the rat after an infection-induced invasion of epidermal cells from the meatal skin. An infection-induced-cornifying metaplastic lesion of the middle ear epithelium revealed nonepidermal cytokeratin expression. The results of this combined study suggested that the cholesteatoma specimens studied had an epidermal origin. The expression of nonepidermal cytokeratins was considered to result from a state of hyperproliferation rather than from metaplasia.


Cytokeratins can be defined as the presence of a cornifying squamous epithelium in the middle ear cavity. It can be associated with the destruction of bone. The cause of cholesteatoma is still a matter of debate, because clinical and histopathological observations have not always produced conclusive evidence of the site of origin, but it is generally accepted that individual cases of cholesteatoma have different causes. Epidermal cells from the external meatus (including the tympanic membrane) can invade the middle ear through preexisting perforations, retraction pockets, or inward proliferation of basal epidermal cells; or the cause can be iatrogenic as a consequence of tympanoplasty. Alternatively, squamous metaplasia of the middle ear epithelium and congenital epidermoid rests in the middle ear have also been proposed as possible sources of cholesteatoma.

To our knowledge, the mechanism that triggers this behavior of epithelial cells has not yet been elucidated, but inflammation and eustachian tube obstruction are assumed to be the most likely candidates.

A new approach that can be helpful in the study of cholesteatoma development is the identification of their intermediate-filament proteins. Intermediate-filament proteins are important constituents of the intracellular cytoskeleton with a typical tissue-specific distribution pattern. In epithelial cells, the intermediate filaments (tonofilaments) are composed of cytokeratins.

In humans, 19 different cytokeratin types can be divided into two major groups of basic and acidic proteins that combine in pairs. In one individual cell type, 2 to 10 individual cytokeratins can occur. Their expression has been shown to depend on the type of epithelium, the stage of differentiation, the functional state, and environmental conditions. According to these properties, cytokeratin expression can contribute to a better understanding of the origin of cholesteatoma, while possible changes in the cytokeratin

<table>
<thead>
<tr>
<th>Source, y</th>
<th>Antibody</th>
<th>Specificity for Cytokeratin No.</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Ramaekers et al., 1983</td>
<td>pKer</td>
<td>Several</td>
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</tr>
<tr>
<td>Ramaekers et al., 1987</td>
<td>RCK 102</td>
<td>5 + 8</td>
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<tr>
<td></td>
<td>RCK 103</td>
<td>Several</td>
<td>1:2</td>
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<tr>
<td></td>
<td>RCK 105</td>
<td>7</td>
<td>1:3</td>
</tr>
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<td></td>
<td>RK50 60</td>
<td>10</td>
<td>1:2</td>
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<tr>
<td>Broers et al., 1986</td>
<td>CK 18-2</td>
<td>18</td>
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<td>Lane et al., 1985</td>
<td>LP9K</td>
<td>19</td>
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</tr>
<tr>
<td>van Mulken et al., 1986</td>
<td>1C7</td>
<td>13</td>
<td>Undiluted</td>
</tr>
<tr>
<td></td>
<td>6B10</td>
<td>4</td>
<td>Undiluted</td>
</tr>
<tr>
<td>Schaafmam et al., 1989</td>
<td>M20</td>
<td>8</td>
<td>1:5</td>
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profile of meatal skin invading into the middle ear cleft can be established.

In this study, we examined the cytotkeratin expression patterns of experimentally induced epidermoid formations in the middle ear of the rat. The results obtained from this experimental study were related to the cytotkeratin profiles observed in human cholesteatoma, external meatal skin, and middle ear mucosa.

MATERIALS AND METHODS

For the induction of purulent otitis media, 20 adult Wistar rats (body weight, about 170 g) from a Mycoplasma pneumoniae-contaminated strain that displayed clinical symptoms like sneezing and rhinorrhea were used. The animals were checked otoscopically for the absence of otitis, and eustachian tube obstruction was performed according to the method described by Kuijpers and van der Beek by electrocautery with the rats under fentanyl citrate (Hypnorm) (intramuscularly, 0.05 mL/100 g of body weight) and diazepam (intraperitoneally, 0.05 mL/100 g of body weight) anesthesia. The condition of the middle ear was checked by otoscopy on days 1, 2, 4, and 8 after the operation and subsequently at weekly intervals.

After survival times varying from 1 week up to 6 months, the animals were killed by an intracardiac injection of pentobarbital sodium (Nembutal) and decapitated. The middle ear and part of the external meatal skin were dissected and processed for paraffin embedding after fixation in 4% formaldehyde solution in 0.1-mol/L phosphate buffer (pH, 7.4) at 4°C for 2 days; this was followed by decalcification in edetic acid (EDTA) (10%; pH, 7.4) for 2 weeks or immediate storage in a decalcification solution (10% edetic acid and 7.5% polyvinylpyrrolidone in 0.1-mol/L TRIS-hydrochloric acid buffer; pH, 7.4) at 4°C for 2 weeks. After rinsing in the same solution without edetic acid for 2 hours, these samples were frozen in liquid nitrogen and cryosectioned at 7 µm. Sections were collected on poly-L-lysin-coated slides, air dried, and stored at -70°C until use.

For immunocytochemistry, the sections were fixed in acetone (at 4°C for 10 minutes), rinsed in phosphate-buffered saline solution (PBS), and incubated with different monoclonal antibodies with known specificities for human cytokeratin, either as an undiluted culture supernatant or diluted in PBS for 45 minutes (for specifications and references, see Table 1). The sections were washed in PBS and incubated with horseradish peroxidase-conjugated rabbit anti-mouse (or swine anti-rabbit for the polyclonal serum pKer) immunoglobulin that contained 5% (vol/vol) rat serum for 30 minutes. After washing in PBS and sodium acetate buffer (pH, 4.9) for 5 minutes, peroxidase activity was detected by using a mixture composed of 0.025% 3-aminon-9-ethylcarbazole, 5% dimethylformamide, and 0.01% hydrogen peroxide in sodium acetate buffer (pH, 4.9). The sections were counterstained with Mayer’s hemalum and mounted in glycerin jelly. As a control, the primary antibody was omitted. Comparing...
Fig 2.—Immunohistochemical staining of rat healing tympanic membrane perforation with the monoclonal antibodies RCK 103 (A) and RKSE 60 (B). Note the selective absence of RKSE 60 staining at the edges of the healing membrane (arrows) (original magnification ×35).

Fig 3.—Immunohistochemical staining shows the cytokeratin profile of infection-induced invasion of meatal epithelium into the rat middle ear cavity with the monoclonal antibodies RCK 103 (A), RCK 102 (B), RKSE 60 (C), and IC7 (D). The left panels show surveys, and the right panels show higher magnifications of the invading epithelium (arrowheads in left panels). Note the differential staining of the skin of the external meatus (E) and middle ear epithelium (M), the focal staining with IC7, and the absence of staining at the invading front with RKSE 60. P indicates tympanic membrane perforation (original magnification, left panels ×10; right panels ×35).

Results

Normal Rat Ear

In the normal rat, a positive staining of the external meatal skin, including the sebaceous glands, hair follicles, and the outer layer of the tympanic membrane, was found with the antibodies pKer and RCK 103. With RKSE 60, only the suprabasal cells were positive, while staining with RCK 102 was limited to the inner layer of the root sheath of the hair follicles (Fig 1). All other antibodies tested were negative.

The middle ear epithelium showed a homogeneous staining with the antibodies pKer, RCK 102, RCK 103, LP2K, and CK 18-2, both in the flat squamous epithelium and in the mucociliary tracts. With RCK 105 and 6B10, the squamous epithelium revealed a scattered positive reaction. With RCK 105, only a subpopulation of the cells in the mucociliary tracts was stained, while no reaction was found with 6B10. The antibodies RKSE 60 and IC7 failed to show any reaction in the middle ear epithelium. With the antibody M 20, no reaction at all was established in rat epithelia. The data obtained are summarized in Table 2.

Eustachian Tube Obstruction

In all ears with obstructed eustachian tubes, an accumulation of purulent fluid was observed otoscopically within 2 days after the operation, followed by a spontaneous perforation of the tympanic membrane and otorrhea between 2 and 8 days. In the majority of the ears, spontaneous closure of the perforation was seen during the second week. In six ears, otorrhea persisted for longer periods, and in two of
Hese rats, killed after 4 and 11 weeks, respectively, sections displayed the invasion of epidermal cells from the metal skin into the middle ear cavity. The epithelium migrated on top of the infection-induced fibrous tissue. The latter contained the original middle ear epithelium, visible as cystlike structures.

Immunocytochemistry demonstrated that the cytokeratin expression in the availing epithelium was identical to that in the native metal skin, except for the frontal area, where staining with RKSE 60 was absent. Remarkably, in this area, the antibodies 1C7 and 6B10, which are not expressed in the metal skin, showed a distinct reaction with the suprabasal epithelial cells (Fig 2). A comparable phenomenon was seen at the edges of tympanic membrane perforations in the ears in which the membrane had started to heal. This is illustrated for RCK 103 and RKSE 60 in Fig 3.

No fundamental difference was seen between the cytokeratin profile in the residual middle ear epithelium and the normal ear, except for the occasional absence of cytokeratin 7 expression, as revealed by RCK 105.

In the ears in which the tympanic perforations were found to be healed, the middle ear cavity revealed a varying amount of fibrous tissue that enclosed the original middle ear epithelium and a central core of pus. On top of this tissue, signs of the formation of a new epithelial layer could be observed locally. In three animals that survived for 4 months after eustachian tube obstruction, isolated areas of stratified squamous epithelium were found. In two ears, this epithelium consisted of noncornifying epithelium, and in the third ear, it clearly demonstrated cornification with a de-squamating stratum corneum, a stratum granulosum that contained keratohyaline granules, and a stratum lucidum. Incubation of sections from this ear with the polyclonal antibody pKer showed a homogeneous staining of this epithelium and the metal skin. The antibody RCK 102 displayed a marked positive reaction in the suprabasal layers of the stratified epithelium (Fig 4). In the external metal skin, only the inner layer of the root sheath of the hair follicles stained positive. Because it concerned paraffin-embedded specimens, no other cytokeratin antibodies could be tested, because their epitopes were destroyed by this procedure.

A specimen embedded in paraffin, obtained from an animal that survived for 4 weeks, displayed a total perforation of the tympanic membrane and a dry middle ear cavity that was completely lined with squamous epithelium, continuous with the mental skin. In this case, testing with the antibody RCK 102 failed to show any reaction with the invaded epithelium.

**Human Specimens**

The cytokeratin profiles observed in specimens from normal human mental skin and middle ear epithelium are summarized in Table 2. They revealed a comparable differential expression to that observed in the same tissues of the rat. The simple epithelium of the middle ear stained homogeneously with the whole panel of antibodies tested, with the exception of RKSE 60, while only scattered cells stained with 6B10 and 1C7. In contrast to the rat middle ear, the antibody M 20 showed a homogeneous positive reaction. The epithelium of the mental skin showed a positive reaction with the polyclonal antibody pKer and the monoclonal antibodies RCK 102 and RCK 103, including hair follicles and sebaceous glands. The antibody RKSE 60 only stained the suprabasal epithelial cells (Fig 5).

The histological appearance of the cholesteatoma specimens was largely divergent with respect to the thickness of the epithelium, the infiltration of epithelial cells into the perimatrix, and the presence and number of inflammatory cells in the perimatrix. The cytokeratin profile, displayed by all 10 cholesteatoma specimens studied, was generally similar to that observed in the mental skin, except for the advancing front at the junction with the middle ear epithelium. At the margin of this area, staining with RKSE 60 was almost completely absent or had shifted toward more superfi-
epithelium, a positive reaction was found in this area. This reaction was strongest at the margin of the advancing front and diminished toward more distant areas (Fig 6). In these specimens, a comparable focal disturbance of the cytokeratin expression was found far distant from this invading front (Fig 5).

COMMENT

The results presented in this study demonstrate a distinct divergence of cytokeratin expression patterns between external meatal skin and middle ear epithelium, both in humans and in rats. This is in line with the general rules for cytokeratin expression in simple and complex epithelia and with the cytokeratin profiles established in skin and respiratory epithelia. Comparison of these cytokeratin-staining patterns, displayed by meatal skin and middle ear epithelium in humans and in rats, demonstrates a close similarity in reaction patterns between these species, with only minor differences for antibodies M 20 and RCK 102. Apparently M 20 does not react with a cytokeratin epitope in the rat. This assumption is further supported by the observation that the epithelia in the nasopharynx were also negative. Although antibody RCK 102 does react with rat cytokeratin epitopes, its staining reaction in the meatal skin is virtually limited to the inner layer of the root sheath of the hair follicles.

The similarity between the cytokeratin patterns of the cholesteatoma matrix and the meatal skin corresponds with the findings made by van Blitterswijk and Grote and Broekaert et al., and provides strong evidence that these cholesteatoma lesions had an epidermal origin. However, the focal expression of cytokeratins 4 (6B10), 13 (1C7), and 19 (LP2K), together with a disturbed cytokeratin 10 (RKSE 60) expression in the cholesteatoma matrix, is at variance with this assumption and needs further explanation. These are nonepidermal cytokeratins. Cytokeratin 19 is preferentially found in simple epithelia and in the basal compartment of some noncornifying stratified epithelia, while cytokeratins 4 and 13 are mainly restricted
noncornifying squamous epithelium. Based on the focal expression of non-
epidermal cytokeratins in the cho-
estaA on matrix, together with the
cornification of cultured middle ear epithelium observed in vitro, von Blit-
terswijk and Grote did not exclude a 
metaplastic origin of cholesteatoma. On the other hand, Broekaert et al. 
considered this focal expression of nonepidermal cytokeratins to be a 
hyperproliferative state as encountered in other hyperproliferative epidermic 
diseases. The present experimental study on the rat can probably contribu-
te to a better understanding of the origin and behavior of ectopic cornifying 
epithelium in the middle ear cavity.

The study shows that purulent otitis media associated with a tympanic 
membrane perforation can lead to the 
vasion of meatal skin into the middle 
ear cavity. This corresponds with com-
parable studies on guinea pigs. These observations favor the 
assumption that infection can be consid-
ered to be an important trigger in the 
eductive expansion of cornifying epithe-
lum into the middle ear cavity. The 
invading epithelium from the meatus 
generally retains its original cytokeran-
atin profile. Strikingly, however, at the 
invading front, a local expression of 
nonepidermal cytokeratins (Nos. 4 and 
13) was noticed, while cytokeratin 10 (RKK6 60) expression, which is a reli-
able marker of cornification, was ab-
sent. A comparable phenomenon was observed at the perforation edges of the 
healing tympanic membrane, at the 
advancing front of the cho-
estaA matrix, and also focially 
distant from this area. This points to 
locally altered terminal differentiation. Therefore, the focal expression of 
nonepidermal cytokeratins in cho-
estaA lesions and the aberrant 
behavior of cytokeratin 10 expression can be explained by assuming that 
these findings were caused by local 
differences in the state of differentiation, correlated with hyperprolifera-
tion, rather than by a metaplastic or-\nigin. In this context, ears with cho-
estaA may have often been treated 
before surgery for a long period with 
cartilage drops that contain corticosteroids. Therefore, a drug-induced change in 
the condition of the epithelium, includ-
ing the cytokeratin profile, cannot be 
excluded. Eichner et al. showed such 
changes in cytokeratin expression patterns with hydrocortisone in tissue 
culture. 

Apart from an epidermal genesis, the presence of an isolated area of 
cornifying epithelium, in one of the 
rats studied, favors metaplasia of the middle ear epithelium as an alter-
native source of cornifying epithelium in the middle ear as was suggested by 
Sadé et al. Especially the staining of this area with the antibody RCK 102, 
which also stains the middle ear epithe-
lum but not the meatal skin, provided conclusive evidence for a 
metaplastic origin in one animal. Such 
a genesis of cholesteatoma, suggested to 
account for about 4% of the total number encountered, is difficult to prove clinically, because these lesions will usually remain unnoticed until the 
tympanic membrane perforates and infection occurs. This makes them in-
distinguishable from a cholesteatoma that originates from an ingrowing 
edermis. The present study dem-
strates that the cytokeratin expres-
sion seems to be very useful to differ-
entiate between these types of cho-
estaA.

Summarizing, this immunohis-
tochemical study demonstrates that 
on account of the similarity among 
the cytokeratin profiles of meatal skin, in-
vading epithelium, and cholesteatoma, 
but can be concluded that the 
cho-
estaA specimens studied had an 
edermal origin. The focal presence of 
nonepidermal cytokeratins in the cho-
estaA matrix may refer to hyper-
proliferative activity rather than to a 
metaplastic origin. In addition, the 
results of this experimental study on the 
support the assumption that corn-
ifying epithelium can arise from the 
middle ear epithelium by an infection-
induced metaplasia.

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