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Keratinocyte Differentiation in Acquired Cholesteatoma and Perforated Tympanic Membranes

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Objective: To evaluate the type of differentiation of keratinocytes of acquired cholesteatoma and its significance for cholesteatoma invasiveness.

Design: Forty acquired cholesteatomas and 10 tympanic membranes with persisting perforations were snap frozen and processed for immunohistochemical studies. Cytokeratin antibodies that represented all subgroups and antibodies that were directed against collagen components of the basal lamina were applied. Expression of these constituents was scored by using light microscopy.

Results: The phenotype of the matrix was generally characterized by an extension of expression of basal cell cytokeratin 14 and hyperproliferation-associated cytokeratins 6, 16, and 17 into the suprabasal cell layers, while the expression of keratinization marker cytokeratin 10 was down-regulated. These features varied greatly at different sites of the matrix and were most marked at the advancing front of the cholesteatoma. A comparable expression pattern, but less pronounced, was observed at the epidermal front of the mucocutaneous junction of the tympanic membrane perforations. This phenomenon was invariably associated with a mononuclear cell infiltrate in the dermis at both junctions. The basal lamina was always intact.

Conclusions: Acquired cholesteatomas show hyperproliferative features. There is a striking similarity between the pronounced expression of this phenotype and the associated inflammation at the mucocutaneous junctions of cholesteatomas and tympanic membrane perforations and those that are observed after epidermal injury. This indicates that epidermis and middle ear epithelium do not form stable junctions and the front can be considered to be a persisting epidermal defect. This involves the permanent presence of "activated keratinocytes" in the junction area that will lead to proliferation and migration, when additional triggers are present.

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In otorhinolaryngology, the differentiation-dependent expression patterns of the cytokeratins (Cks) \(^1\) \(^4\) have been used to shed light on the continuing discussion about whether cholesteatomas originate exclusively from the meatal epidermis or whether they can also be the result of squamous metaplasia of the middle ear epithelium. The expression of mainly epidermal Cks in cholesteatoma was generally considered to reflect an epidermal origin.\(^5\) \(^8\) The focal expression of nonepidermal Cks observed in some specimens has been explained in different ways. Broekaert et al\(^9\) and Vennix et al\(^7\) explained that the expression of these "ectopic Cks" could be the result of a local state of hyperproliferation or the result of the modulating effect of the middle ear mesenchyma,\(^9\) while van Blitterswijk et al\(^10\) have considered metaplasia as the possible cause.

During recent years, hyperproliferation-associated Ck 16 has been studied in an attempt to explain the expansive behavior of cholesteatomas.\(^10\) \(^12\) However, the results of these studies varied considerably, even when the same antibodies were applied. These discrepancies have to be ascribed to the application of poorly characterized antibodies\(^11,12\) and to the study of ill-defined parts of the matrix. In addition, the mere presence of Ck 16 in cholesteatoma does not necessarily indicate a hyperproliferative condition of the matrix, because this Ck has also been demonstrated in normal deep meatal skin.\(^13,14\)

In a recent study,\(^14\) we observed a mixed differentiation phenotype in deep meatal skin that is not found in normal
MATERIALS AND METHODS

All the specimens that were used in this study were obtained during ear surgery. These specimens comprised (1) 10 tympanic membranes with persisting partial or subtotal perforations from dry ears without cholesteatoma and (2) 40 acquired cholesteatomas of varying size with tympanic membrane perforations.

Immediately after dissection, the specimens were frozen in liquid nitrogen and stored at −70°C. Cryostat sections (7 μm) were placed on poly-L-lysine-coated slides, dried in a cold airstream for 30 minutes, and stored at −70°C until required. Immunostaining was performed by using a previously described indirect immunoperoxidase technique. The antibodies that were applied and their characteristics are indicated in the Table.

RESULTS

CHOLESTEATOMA MATRIX

The matrix consisted of keratinizing epithelium with local variations in thickness. The underlying stroma was composed of loose connective tissue that contained capillaries and varying numbers of lymphocytes, plasma cells, and macrophages. These features varied considerably between specimens and within the same specimen.

The Ck expression patterns showed variations within and between specimens (Figure 1). These patterns could roughly be divided into the 3 types that are presented diagrammatically in Figure 2.

Type 1 showed homogeneous expression of basal cell Cks 5 and 14 in the basal cell layer, while Ck 14 expression could also extend into 1 or more suprabasal layers. Incidentally, the basal cells showed sparse expression of Ck 19. All suprabasal cell layers showed expression of keratinization marker Ck 10. Hyperproliferation-associated Cks 16 and 17 revealed focal to heterogeneous expression in the parabasal cell layer, while Ck 6 was homogeneously expressed in a varying number of suprabasal layers and in nearly all basal cells. In the stroma, no or only a few scattered inflammatory cells were observed.

In the type 2 profile, Ck 19 was completely absent. Cytokeratin 5 was expressed in scattered basal cells, while the other basal cell marker Ck 14, besides being expressed in the basal cells, extended into all suprabasal cell layers. Hyperproliferation-associated Ck 6 was expressed in all cell layers, while Ck 16 and 17 were found in a varying number of suprabasal cell layers; Ck 17 expression was usually less marked than that of Ck 16. There was no expression of keratinization marker Ck 10 in 1 or more suprabasal cell layers. In the underlying stroma, scattered inflammatory cells were always observed.

In the type 3 profile, Ck 19 and basal cell Ck 5 were not expressed, while the other basal cell marker Ck 14 and hyperproliferation-associated Ck 6 were expressed in all cell layers, and Cks 16 and 17 were expressed in all suprabasal cell layers, with Ck 10 absent or only expressed in the outermost cell layers. In 2 of 40 cholesteatoma specimens, limited areas of the matrix showed scattered keratinocytes that expressed the stratification markers of noncornifying epithelia (ie, Cks 4 and 13), as well as vimentin. This profile, which was only observed rarely in the matrix, was often associated with distinct basal cell hyperplasia, parakeratosis, dense accumulations of inflammatory cells, and many capillaries in the stroma. These latter features were detected with vimentin and collagen IV staining. The staining patterns for collagens IV and VII indicated that the basal lamina was always intact.

CHOLESTEATOMA FRONTS

The epidermal fronts that were found in the large majority of specimens either penetrated into the stroma (Figure 3, A) or were situated on top of it in contact with the middle ear epithelium (Figure 3, B and C; Figure 4; and Figure 5, C). They showed a varying degree of hyperplasia and dysplasia and papillary ingrowth into the stroma and were frequently found to have undermined the middle ear epithelium (Figure 3, B; Figure 4, K-N; and Figure 5, C) or to have migrated on top of it. The adjacent middle ear epithelium showed hyperplasia (Figure 4 and Figure 5, C). Morphologically harmonious junction areas that showed only slight hyperplasia were also observed incidentally (Figure 3, C). The lamina propria at the junction areas contained many capillaries and invariably showed the presence of large accumulations of inflammatory cells that were mainly located ahead of the front (Figure 5, A) and often were associated with local disruption of the middle ear epithelium (Figure 3, B).

Based on their Ck expression profile, 2 different phenotypes were identified in the advancing fronts that could both be present within the same specimen. The first phenotype (Figure 4, A, C, E, G, I, K, and M) showed a similar Ck expression pattern to that of the type 3 profile of the cholesteatoma matrix. Ck 19 (Figure 4, M) and Ck 5 were absent, but all cell layers...
was no Ck 16 expression. The antibodies that were compared with those of the normal epithelium. There was increased expression of basal cell markers Cks 5 and 14, the stratification markers Cks 4 and 13, and hyperproliferation-associated Cks 6 and 17 in comparison with those of the normal epithelium. There was no Ck 16 expression. The antibodies that were directed against collagen types IV and VII revealed that the basal lamina was continuous in all the specimens (Figure 5, A).

The expression patterns of Cks 6, 16, and 14 (Figure 4, H, F, and B, respectively) in the second phenotype (Figure 4, B, D, F, H, J, L, and N) were similar to those observed in the first phenotype, but the upper cell layers expressed both keratinization marker Ck 10 (Figure 4, D) and the stratification markers of noncornifying epithelium Cks 4 and 13 (Figure 4, L and J, respectively). In addition, the simple epithelial cell marker Ck 19 (Figure 4, N) was expressed heterogeneously in the basal and suprabasal cell layers. Cytokeratin 6 was absent in the Ck 19–positive basal cells, while scattered cells that expressed simple epithelial Cks 7, 8, and 18 were observed incidentally in the superficial layers. Staining with vimentin antibodies was observed in scattered keratinocytes in 2 specimens. Morphologically, the junction areas of the second phenotype were characterized by the presence of dysplasia.

In the junction area of the cholesteatoma front, the hyperplastic middle ear epithelium showed an increased expression of basal cell markers Cks 5 and 14, the stratification markers Cks 4 and 13, and hyperproliferation-associated Cks 6 and 17 in comparison with those of the normal epithelium. There was no Ck 16 expression. The antibodies that were directed against collagen types IV and VII revealed that the basal lamina was continuous in all the specimens (Figure 5, A).

In 6 specimens of the 10 tympanic membrane perforations that were studied, the epidermis ended at some distance from the perforation edge, and the middle ear epithelium was covering part of the lateral surface of the tympanic membrane (Figure 6, B). In 4 specimens, the mucocutaneous junction coincided with the perforation edge (Figure 6, A).

Figure 1. Survey of cholesteatoma matrix shows widely divergent expression patterns of the hyperproliferation-associated cytokeratin 16 at different sites (×50).

**TYMPANIC MEMBRANE PERFORATIONS**
showed basal projections into the connective tissue (Figure 6). The adjacent middle ear epithelium was composed of a normal flat to cuboidal epithelium, which could be clearly distinguished from the epidermal cells on the basis of their different Ck expression patterns. There was a gradual transition between both types of epithelium in all of the specimens (Figure 6, C). No undermining or overgrowth of the middle ear epithelium by the epidermis was observed. At the mucocutaneous junction, the lamina propria invariably contained a distinct accumulation of mononuclear inflammatory cells (arrows in Figure 6, A, B, and D) and many capillaries (Figure 5, B).

The epidermal front revealed a weak focal to heterogeneous expression of basal cell Ck 5 (Figure 6, E), while Ck 14 was expressed in all cell layers (Figure 6, F). Expression of Cks 6, 16, and 17 (Figure 6, H, I, and J, respectively) extended into a varying number of suprabasal cell layers. Cytokeratin 10 (Figure 6, G) was absent in 1 to 2 parabasal cell layers, identical to type 2 Ck expression in the cholesteatoma matrix. The epidermis in the remaining part of the tympanic membrane showed a weak, nearly homogeneous expression of Ck 5 in the basal cell layers and a strong expression of Ck 14 in the basal and parabasal cell layers (Figure 6, E and F, respectively). Cytokeratin 10 was expressed in all suprabasal cell layers (Figure 6, G), while Ck 19 was present focally in the basal cells. Hyperproliferation-associated Ck 6 was expressed in 2 or more suprabasal cell layers (Figure 6, H), while Cks 16 and 17 showed only weak focal expression in the parabasal cell layer (Figure 6, I and J). The Ck profile of the middle

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**Figure 2.** Diagram of the different expression patterns of cytokeratins (Cks) in basal and suprabasal cell layers of cholesteatoma matrix (types 1-3). For comparative purposes, the Ck expression of the normal epidermis and epidermis of cartilaginous and osseous meatus have been included.

**Figure 3.** Immunohistochemical staining of cholesteatoma fronts. A, Cholesteatoma front penetrating into the stroma. Note the expression of basal cell cytokeratin 14 in all cell layers of the front, while expression in the matrix distant from the front is limited to the basal and parabasal cell layers. B, Cholesteatoma front undermining the middle ear epithelium, reactive for cytokeratin 8 (arrowheads). Note the accumulation of inflammatory cells (arrow) at the junction and local disruption of the middle ear epithelium. C, Cholesteatoma front with a morphologically stable mucocutaneous junction and dermal accumulation of inflammatory cells (arrow) immunostained for cytokeratin 10. In comparison with the fronts in A and B, there is less hyperplasia. The keratinization marker cytokeratin 10 is absent in the parabasal cell layer (×50 [A, B], ×180 [D]).
Figure 4. Overview (CX) that shows the distribution of cells (a, b). Two regions are highlighted with dots and arrows. In both regions, a dense population of cells is observed, indicating an area of interest for further study.

Figure 5. A detailed view (CX) of the same area as in Figure 4. The distribution of cells is more defined, with a clear boundary between two distinct populations. The highlighted region shows a higher density of cells, suggesting a region of interest for further investigation.

Figure 6. A magnified view (CX) of a specific region within the highlighted area. The details of cell distribution are more pronounced, and the density of cells is further analyzed to understand the biological significance of this region.

Figure 7. A comparison (CX) of the highlighted region from Figures 5 and 6, showing a consistent pattern of cell distribution. The density and arrangement of cells remain consistent, indicating a stable and significant area for further study.

Figure 8. A cross-sectional view (CX) of the highlighted region, providing a different perspective to understand the spatial arrangement of cells. The pattern of cell distribution is observed in a longitudinal direction, offering insights into the potential function and organization of this region.
expression of both epidermal and hyperproliferation-associated Cks). However, the levels of expression reflect a more hyperproliferative phenotype. The expression of Ck 19 and basal cell Ck 5 is severely reduced or absent in the basal cells of the cholesteatoma matrix. In the suprabasal cells, there is down-regulation of keratinization marker Ck 10 and up-regulation of basal cell Ck 14 and hyperproliferation-associated Cks 6, 16, and 17. These changes in the Ck profile indicate an altered process of differentiation and result in a phenotype that is similar to that observed after skin injury,27 in hyperproliferative skin disease,28,29 and in malignant transformation.1,28 The Ck pattern indicates the presence of a hyperproliferative condition, but it can differ widely between individual specimens and at various sites within the same specimen. The dissociation between the expression of basal cell markers Cks 5 and 14 is remarkable. This phenomenon has also been observed in psoriasis.6

The hyperproliferative phenotype is most pronounced at the advancing front of cholesteatoma. The additional expression of nonepidermal Cks and vimentin in a proportion of the fronts appears to be related to dysplasia. This demonstrates that the presence of nonepidermal Cks, which are also observed incidentally in small areas of the matrix, does not necessarily refer to a metaplastic origin of the cholesteatoma, as suggested previously.6

These events at the advancing fronts indicate severe disorganization of normal epidermal differentiation and proliferation and provide further evidence to support the suggestion that the front area is the most important site of matrix expansion. However, the present observations do not confirm that the expansion is associated with a distortion of the basal lamina.30 In the major part of the matrix outside the front, the hyperproliferative condition is less marked. This indicates that once the matrix has formed, there is a tendency to return to the native phenotype.

The invariable presence of a mononuclear cell infiltrate at the advancing front reflects a close relationship between the hyperproliferative condition and a chronic inflammatory process. The presence of inflammatory cells at the mucocutaneous junction of the cholesteatoma matrix has been reported previously,20 and has been suggested to be owing to the irritating effect of keratin at the junction or to the lack of a tight seal, allowing microorganisms to enter the dermis. It was concluded30 that the inflamed stroma, rather than the inherent characteristics of the squamous epithelium, determined the invasive nature of the cholesteatoma.

Although there is no doubt that inflammation or infection are important causal factors in cholesteatoma genesis, the answer to the question of whether epidermis and middle ear epithelium can form a stable junction appears to be of more importance for explaining the invasive nature of cholesteatoma. Animal studies have shown that meatal skin transplants can grow in the middle ear, although differentiation of the keratinocytes seems to be modulated by the stroma of the middle ear.9 However, data that have been reported on the character of the junction between epidermal cells and middle ear epithelium have been contradictory.9,30
Recent studies on retinoic acid perception have led to a better understanding of these phenomena. Retinoids become activated in the presence of retinoic acid and help to form a better understanding of the role of retinoids in gene expression and differentiation.

Detailed studies in the field of retinoic acid perception show that the expression of retinoid-dependent genes is regulated by the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). The activation of these receptors by retinoids leads to the expression of target genes, including those involved in cell differentiation and proliferation.

However, the mechanism by which retinoids interact with these receptors and how this interaction is regulated is still not fully understood. Further research is needed to elucidate the pathways involved in retinoid perception and how these pathways interact with other signaling pathways.

Figure 1: Immunohistochemical staining of mucinous tumors of the eye. The staining is performed using antibodies against mucin and the results are visualized using a fluorescent microscope. The stained sections are then analyzed using ImageJ software to quantitate the amount of mucin.
response to a large variety of injurious stimuli. They then start to express hyperproliferation-associated Cks, change their adhesive properties, and become migratory. In addition, they produce and respond to immunological signals (eg, cytokines and epidermal growth factors). Cytokines can then induce the attraction of inflammatory cells from the circulation and promote their migration toward the site of injury. This cascade of events can also be assumed to occur at the mucocutaneous junctions of tympanic membrane perforations and cholesteatomas. At these sites, it will become a continuing process because epidermal homeostasis cannot be restored.

Although this condition can be considered as the main underlying cause of matrix propagation, the existence of apparently stable junctions at the perforation edge and epidermal expansion at the cholesteatoma front needs further explanation. This study shows that there is no qualitative difference between the reactions at the 2 junctions, but that the hyperproliferative condition and the intensity of the inflammatory process are more pronounced at the cholesteatoma front. Therefore, we must assume that there are additional injurious stimuli that enhance this reaction and disturb the "stable" equilibrium between the epidermis and middle ear epithelium at the cholesteatoma junction. One can only speculate about the nature of these stimuli, which may also be responsible for the induction of the pronounced hyperproliferative phenotype in parts of the matrix. The environmental conditions (eg, humidity, accumulated cellular debris, and desquamation products) in the middle ear that can promote inflammation or infection are the most likely candidates.

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ARCH OTOLARYNGOL HEAD NECK SURG/VOL 122, AUG 1996

832