Growth and Differentiation of Meatal Skin Grafts in the Middle Ear of the Rat

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**Objectives:** To determine the behavior of epidermal cells after transplantation in the middle ear.

**Design:** In a rat model, full-thickness meatal skin grafts were transplanted into the middle ear and studied morphologically and immunohistochemically with the use of antibodies directed against different cytokeratin (CK) polypeptides, which are markers of different types of epithelial cell differentiation.

**Results:** The grafts had either transformed into epithelial cysts or had become integrated into the middle ear epithelium. The epithelium of the integrated grafts showed gradual transition into the epithelium of the middle ear. A clear distinction between epidermal cells and middle ear epithelium could be made only on the basis of their CK profiles. The CK profiles of the grafts revealed a decrease in the expression of epidermal CKs, while nonepidermal CKs became expressed. These changes can be ascribed to replacement of the dermal mesenchyma by mesenchyma from the middle ear. In two ears with superimposed infection, the graft epithelium showed expansive growth.

**Conclusions:** Meatal epidermis is well tolerated in the middle ear, but superimposed infection can induce expansive growth. These findings favor the concept that the progressive growth of cholesteatoma is related to the presence of inflammatory processes.

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**C**holesteatoma is a destructive middle ear disease caused by the progressive expansion of keratinizing squamous epithelium in the middle ear and mastoid. Over the years several theories have been put forward to explain the origin of this epithelium. These refer to the presence of embryonic remnants of squamous epithelium, to squamous metaplasia of the middle ear epithelium, and to the migration of epidermal cells from the external ear canal or tympanic membrane by retraction, by basal cell proliferation, or through a tympanic membrane perforation. Middle ear surgery (tympanoplasty and stapes surgery) and trauma have also been proposed as a possible source for the entrapment of epidermal cells in the middle ear cavity.  

Although infection and eustachian tube dysfunction are generally assumed to be important etiologic factors in cholesteatoma genesis, factors and mechanisms that promote the migration and expansion of epidermal cells into the middle ear are not entirely understood. Many experimental studies and animal experiments have been performed to gain more insight into the pathogenesis of this condition, but the basic aspects still remain obscure.

Migration of epidermal cells into the middle ear has been observed after the application of a large variety of irritating chemicals to the tympanic membrane. However, these conditions are far removed from the natural situation. In a more pathophysiological...
MATERIALS AND METHODS

For this study, adult healthy Wistar rats (body weight about 180 g) were used (institutional guidelines regarding animal experimentation were followed). The animals were anesthetized with fentanyl citrate (Hypnorm) intramuscularly, 0.05 mL/100 g of body weight) and diazepam (intraperitoneally, 0.05 mL/100 g of body weight). The middle ear was reached by a retroauricular incision and a small fenestra was made in the posteroverentral wall of the bulla. Subsequently, a small full-thickness graft (1 to 4 mm²) was dissected from the deep mental skin and implanted into the middle ear cavity after local scarring of the mucosa.

The animals were killed by an intracardiac injection of pentobarbital sodium (Nembutal) after survival times varying from 1 month up to 6 months. For routine light and electron microscopic studies, the whole middle ear was dissected from the skull and fixed in phosphate-buffered (0.1 mol/L; at a pH of 7.4) 2.5% glutaraldehyde and subsequently decalcified in a solution containing 10% edetic acid (disodium salt) and 1.5% glutaraldehyde. After appropriate trimming and dehydration, the specimens were either processed for embedding in glycol methacrylate (GMA) for light microscopic studies or in epoxy resin (Epon) for electron microscopy. The GMA sections (2 μm) were stained with toluidine blue.

For immunohistochemistry, immediately after dissection from the skull, the whole middle ear or the dissected grafts were, immediately after dissection from the skull, stored in a decalcification solution containing 10% edetic acid (disodium salt) and 7.5% polyvinyl pyrrolidone in 0.1 mol/L of TRIS-hydrochloride buffer (at a pH of 7.2) at 4°C for a period ranging from 4 to 6 days. The specimens were then rinsed in the same solution without edetic acid, frozen in liquid nitrogen, and stored at −70°C until required. Cryosections (7 μm) were placed on poly-L-lysine-coated slides, air dried, and fixed in acetone (−4°C; 10 minutes). Immunohistochemical staining of these sections was performed as described previously.20

The specifications of the antibodies applied are given in Table 1. In addition to monoclonal antibodies directed against different Ck polypeptides, antibodies directed against vimentin, which is the intermediate filament protein of mesenchymal cells, were included in this study. Most of the antibodies have been characterized for their specificity to human Cks, numbered 1 through 20 by Moll et al.21,22 When indicating a certain Ck polypeptide by the number used in the Moll et al catalog, we refer to the rat analogue of its human counterpart.

<table>
<thead>
<tr>
<th>Table 1. Antibodies</th>
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<tbody>
<tr>
<td>Antibody</td>
</tr>
<tr>
<td>RCK 103</td>
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<tr>
<td>RCK 102</td>
</tr>
<tr>
<td>RCK 105</td>
</tr>
<tr>
<td>E2</td>
</tr>
<tr>
<td>CK 18-2</td>
</tr>
<tr>
<td>LP2K</td>
</tr>
<tr>
<td>RCK 107</td>
</tr>
<tr>
<td>LL002</td>
</tr>
<tr>
<td>RKSE 60</td>
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<td>6b10</td>
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<tr>
<td>1C7</td>
</tr>
<tr>
<td>KA 12</td>
</tr>
<tr>
<td>RV 203</td>
</tr>
<tr>
<td>V9</td>
</tr>
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*CK indicates cytokeratin.
†E2 was supplied by Sergey M. Tryanowsky, PhD, Moscow, Russia.
‡KA 12 was supplied by Raymond Nagle, PhD, Tucson, Ariz.

approach, the ingrowth of mental skin into the middle ear was observed after the instillation of bacteria into the guinea pig middle ear cavity13 and after eustachian tube obstruction in rats with severe upper respiratory tract infection.29 In an experimental study on the cat, Jackson and Lim16 transplanted free mental skin grafts into the middle ear to study the interaction between epithelium and mucous membrane at the transition zone. They obtained a rejection rate of about 30%. The epithelium of surviving grafts revealed labile junctions with middle ear epithelium.

In this study, a comparable experimental design was used. Free mental skin grafts were transplanted into the rat middle ear. In an attempt to better define the behavior of the mental skin keratinocytes in the middle ear cavity, the expression of cytokeratin (Ck) polypeptides was studied, in addition to routine light and electron microscopy.

Cytokeratins are a class of intermediate filament proteins that are exclusively present in epithelial cells.21,22 They are important markers of cell differentiation and are distributed in special combinations, depending on the type of epithelium, the location of epithelial cell within the epithelium, and the stage of development.21–24

The 20 different human keratins have been cataloged by Moll et al21,25 and numbered 1 through 20. In general, simple epithelia express Cks 7, 8, 18, and 19, while stratified and complex epithelia express Cks 5 and 14 in their basal layers. Cornified epithelia express Cks 1, 2, 10, and 11 in the suprabasal layers, while in noncornifying epithelia, Cks 4 and 13 are found. In addition, hyperproliferative epithelia can express Cks 6 and 16.

These expression patterns may change during disease processes,20 (pre)malignant transformation,37 in tis-
Table 2. Cytokeratin Expression of Meatal Skin Grafts*

<table>
<thead>
<tr>
<th>Months</th>
<th>Form</th>
<th>Broadly Reacting</th>
<th>Simple Epithelial Cytokeratins</th>
<th>Stratified Epithelial Cytokeratins</th>
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<tr>
<td></td>
<td></td>
<td>RCK 103</td>
<td>RCK 102 E2 CK 18-2 19</td>
<td>RCK 107 LL002 RKS 60 6B 10 127 6</td>
</tr>
<tr>
<td>1</td>
<td>Integrated</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1.5</td>
<td>Integrated</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Integrated</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Integrated</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>Cyst</td>
<td>+</td>
<td>h</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Cyst</td>
<td>+</td>
<td>h</td>
<td>h</td>
</tr>
<tr>
<td>4</td>
<td>Cyst</td>
<td>+</td>
<td>h</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>Infected</td>
<td>+</td>
<td>h</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Infected</td>
<td>+</td>
<td>h</td>
<td>+</td>
</tr>
<tr>
<td>Meatal epithelium</td>
<td>+</td>
<td>+</td>
<td>h</td>
<td>+</td>
</tr>
<tr>
<td>Middle ear epithelium</td>
<td>+</td>
<td>+</td>
<td>h</td>
<td>-</td>
</tr>
</tbody>
</table>

*Plus sign indicates homogenous staining; h, heterogeneous staining; f, focal staining; and minus sign, negative.
†Negative at front.

sue culture, and during wound healing. The presence of the Ck polypeptides can be demonstrated immunohistochemically with the use of monoclonal antibodies directed against the individual Ck polypeptides.

RESULTS

From a total number of 17 transplanted meatal skin grafts, 16 could be recovered after an observation period varying from 1 month to 6 months. Infections of the middle ear, which persisted up to the time of death, developed in two ears shortly after transplantation. These ears together with 10 noninfected and two normal ears were used for immunohistochemistry (Table 2). Apart from this, two implants (survival times 2 and 4 months, respectively) were used for light microscopic studies (GMA sections) and two (survival times 2 and 6 months, respectively) were used for ultrastructural studies.

LIGHT AND ELECTRON MICROSCOPY

Histological sections revealed that in the noninfected ears, five small grafts had become transformed into epidermal cysts. They were embedded in a thickened area of the lamina propria and were completely surrounded by fibrous tissue, which was lined by the middle ear epithelium on the side of the cavity. The remaining nine grafts had become integrated into the middle ear mucosa and there was continuity between the graft epidermis and the middle ear epithelium. The epithelium of the original graft could be recognized by the presence of the skin appendages. Generally, the epithelium of the grafts in the noninfected ears had retained its original morphological features (Figure 1). At some sites the keratinocytes appeared to be less regularly arranged, and the number of cell layers sometimes varied from site to site. In the epidermal cysts, the newly formed epithelium was mainly composed of two cell layers that predominantly showed parakeratosis.

In the GMA sections, no clear demarcation could be established between the epithelium of the graft and the middle ear epithelium. At the sites where the cut edge of the graft could still be recognized, there was a fairly abrupt transition from stratified squamous epithelium to flattened epithelium that had covered the bare surface of the dermis. This epithelium was continuous with the middle ear epithelium (Figure 1). Electron microscopy showed that the features of the epithelium that covered the bare surface of the dermis differed locally. Close to the original stratified epithelium of the graft, the flat epithelium had the features of epidermal cells with many desmosomes and hemidesmosomes. Further away from this site, these structures gradually disappeared and an increasing number of cells revealed features of the middle ear epithelium with secretory granules and/or pinocytosis (Figure 2). The lamina propria of the grafts occasionally contained few lymphocytes and scattered mast cells.

IMMUNOHISTOCHEMISTRY

The data obtained from the immunohistochemical studies are summarized in Table 2 and depicted in Figure 3 through Figure 6.
NORMAL EPITHELIUM

Immunohistochemical staining patterns showed a positive reaction with the broadly reacting antibody RCK 103 in all layers of the meatal epidermis, including the skin appendages (Figure 3, A). With the Ck 10 marker of keratinizing epithelium (RK56 60), only the suprabasal cell layers of interfollicular epidermis stained (Figure 3, B). Staining with the Ck 14 basal cell markers RCK 107 (Figure 3, C) and LL002 was limited to the basal cells in the interfollicular epidermis and skin appendages. The antibody RCK 102 (Cks 5+8) only reacted with the inner root sheath of the hair follicles, while the hyperproliferation-related Ck 6 marker (KA 12) only stained the sebaceous glands (Figure 3, D). None of the other Ck antibodies applied in this study reacted with the normal meatal skin. Staining with the vimentin antibodies RV 203 and V9 was limited to the intraepithelial dendritic cells and the dermal connective tissue cells.

The middle ear epithelium showed a homogenous reaction with the broadly reacting Ck antibody RCK 103; with RCK 102 (Cks 5+8); and with the simple epithelial markers Ck 18 (Ck 18-2), Ck 19 (LP2K), and Ck 8 (E2). A heterogeneous reaction was observed with the Ck 7 antibody (RCK 105) and with the Ck 4 marker of nonkeratinizing squamous epithelium (6B10). The Ck 14 antibodies RCK 107 and LL002 that are directed against basal cell Cks revealed a distinct staining of the basal cells. Staining with the vimentin antibodies was limited to the fibroblasts of the lamina propria, intraepithelial lymphocytes, and to some epithelial cells in the mucociliary tract near the tympanal orifice of the eustachian tube.

EPIDERMAL GRAFTS

Integrated Grafts

Figure 4 shows micrographs of the immunohistochemical staining patterns of an integrated graft removed after 2 months using various antibodies. All the epidermal grafts that had become integrated into the epithelial lining of the middle ear revealed homogeneous staining with the broadly reacting antibody RCK 103 (Figure 4, A). The suprabasal cells showed homogeneous staining with the Ck 10 marker of keratinizing epithelium (RK56 60) (Figure 4, B), while the basal cells homogeneously stained with the basal cell Ck 14 antibodies RCK 107 (Figure 4, C and D) and LL002. In addition, the epithelium of three of these grafts (the rats had survived for 2, 2, and 4 months, respectively) revealed focal or heterogeneous staining.
munohistochemistry could distinguish clearly between these different cell types because of their dissimilar Ck profiles. The stable integration of the graft epithelium into the epithelial lining of the middle ear is at variance with the observations made by Jackson and Lim\textsuperscript{16,31} in the cat. These authors observed poor stability at the mucocutaneous junction, which was associated with a localized inflammatory reaction. This phenomenon was held re-
sponsible for the finding that the graft epithelium had been replaced by the mucous membrane. They suggested that the skin mesenchymal tissue failed to maintain epidermis in the bulla but could successfully support the mucosal epithelium.

Immunohistochemistry using antibodies raised against human Ck polypeptides demonstrated that these reagents that normally react with the different types of epithelium in humans were also positive in the same type of epithelium in the rat. The specificity of these antibodies for epithelia at other sites in the body of the rat was also found to be comparable with that in the human system. The only difference was observed with the antibody RCK 102 (Cks 5+8), which reacts with the human epidermis but not with the rat epidermis.

In comparison with the normal mental skin, immunohistochemistry showed changes in the Ck profiles of the graft epidermis. There was a decrease in the expression of Ck 10, a specific marker of keratinization. Cytokeratins 4 and 13, which are normally present in the suprabasal layers of nonkeratinizing epithelium but not in interfollicular epidermis, became apparent. In addition, Cks characteristic of simple epithelia were also expressed. These changes, which were most pronounced...
in the epithelium of the cysts, indicated disturbance of the normal skin type terminal differentiation.

Regulation of the growth and differentiation of epithelia is a complex process that depends on genetically determined intrinsic factors as well as extrinsic factors. It has become evident from tissue culture studies and tissue transplantation experiments, including homotopic and heterotopic epithelial mesenchymal recombination, that the intrinsic morphological and biochemical differentiation of adult integumental epidermis can be modulated reversibly by the character of the subepithelial connective tissue that differs from region to region. In the light of these findings, the observations made in the present study can be explained as follows: after the transplantation of meatal skin into the middle ear, the subepithelial connective tissue of the graft will be replaced to a varying extent by connective tissue originating from the middle ear mucosa. This new connective tissue will modulate the differentiation of the epidermis in a different way from that of the original dermal connective tissue and can be held responsible for the changes in Ck expression. It was difficult to trace the extent of connective tissue replacement in the integrated grafts. However, in the epidermal cysts, the newly formed epithelium was fully supported by connective tissue that had originated from the middle ear. This can explain why the changes in Ck expression were most pronounced particularly in these areas. Another factor that cannot be fully excluded is that the changed environmental conditions in relation to gas composition and humidity, which are different in the middle ear, may have had an additional modulating effect.

The nonepidermal Cks mentioned above are not expressed in mature meatal skin but they have been reported to be present during embryonic and fetal development. Their expression is switched off during final maturation. So we can conclude that changed extrinsic factors can induce these cells into renewed synthesis of these Cks.

The lack of expression of Ck 6, considered to be a marker of hyperproliferation in epidermal cells in the grafts in the noninfected ears, demonstrated that meatal skin does not develop hyperproliferative activity when transplanted into the middle ear. One cyst tested for Ck 6 revealed a heterogeneous expression pattern, but this seemed to be related to the presence of inflammatory cells in the stroma. The progressive growth of epidermis in two ears with superimposed infection indicated that such a process can induce enhanced proliferation and migration of the transplanted epithelium. Staining for the hyperproliferation marker Ck 6 was not performed in these cases, but the loss of Ck 10 at the migrating front suggested a state of hyperproliferation, as is found during wound healing. The infection-induced expansion of epidermis in this experimental model supports the concept that inflammatory processes determine the invasive nature of cholesteatoma matrix.

Summarizing, this study shows that in the absence of infection, epidermal cells are tolerated in the middle ear and do not show any abnormal proliferative activity under these circumstances. Superimposed middle ear infection tends to cause the epidermis to expand.

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