Melanocyte localization and distribution in human cholesteatoma

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Summary. Introduction: Melanocytes in skin are derived from the neural crest and colonize the epidermis in the first trimester of gestation. Melanocytes have been observed in the nasopharyngeal, inner ear and oral mucosa and should therefore be present in the middle ear mucosa. Aims: To identify and determine the distribution of melanocytes in human cholesteatoma and normal meatal skin in Caucasian adults. Material and methods: Human cholesteatoma (n=18) and normal meatal skin samples (n=10) were investigated immunohistochemically with anti-HMB-45 and MART-1 antibodies. Localization and distribution of melanocytes were assessed in the epidermis and cholesteatoma using an automatic analyzing system. Results: Regular skin exhibited melanocytes within the epidermis and accounted for 10% of the total cell number. They occurred partly as membrane-bound clusters. Cholesteatoma matrix melanocytes were observed in the basal layer and exhibited an oval or round morphology. Decreased numbers of melanocytes in the basal layer correlated with keratinization within cholesteatoma samples. Melanocytes revealed monomorphic nuclei, abundant cytoplasm containing particles of melanin. Found adjacent to glands and blood vessels, melanocytes were also scattered among the mesenchymal cells. Accounting for 2-6% of the total cell number within the squamous epithelium, melanocyte density was significantly lower in cholesteatoma tissue than in skin. Conclusions: The melanocyte distribution pattern was different when comparing the epithelia of skin and cholesteatoma. The presence of melanocytes in cholesteatoma may be due to an ingrowth, consequently controlled by keratinocyte-derived signals. In terms of the pathogenesis of cholesteatoma, neither squamous metaplasia nor melanocyte metaplasia can be excluded by our data.

Key words: Melanocytes, Immunohistochemistry, Epidermis, Cholesteatoma

Introduction

Melanocytes in skin are derived from the neural crest and they colonize the epidermis in the first trimester of gestation (Zhang et al., 2004). The epidermal melanocyte manifests a distribution in the early differentiating epidermis (Mishima and Widlan, 1996). During embryogenesis, the middle ear mucosa is derived from pharyngeal pouches that also give rise to the nasopharyngeal and oral mucosa derived from oral ectoderm. Melanocytes have been observed in the nasopharyngeal and oral mucosa, therefore they are supposed to be present in the middle ear mucosa (Lin and Zak, 1982; Suder and Bruzewicz, 2004). Middle ear cholesteatoma is characterized by an intrusion of keratinizing stratified squamous epithelium into the middle ear. As soon as the epithelium begins to hyperkeratinize, the destructive behavior of cholesteatoma is triggered (Svane-Knudsen et al., 2004). The pattern of cytokeratins (CKs) expression correlates well with the state of keratinocyte proliferation, migration and differentiation. CKs immunohistochemistry can aid in evaluating of hyperproliferation of cholesteatoma. Cytokeratins used in our previous study have a relatively limited expression profile. Cytokeratins
CK10, CK34BE12 and CK14 are relatively specific for the basal and suprabasal layers of the epithelium. They are considered as keratinizing squamous epithelium that indicates basal keratinocyte hyperplasia (Olszewska and Sudhoff, 2007). The debate about how epidermal structures are established in the middle ear is still controversial (Olszewska et al., 2004). The abnormal behavior of cholesteatoma epithelium seems to be triggered by an immune cell infiltrate releasing large amounts of different cytokines and growth factors. Some of those factors, produced in and released from keratinocytes, may be involved in regulating the proliferation and differentiation of epidermal melanocytes. Very few publications have discussed the presence of melanocytes in the normal middle ear mucosa (Lin and Zak, 1982; Suder and Bruzewicz, 2004). A Medline-Pubmed (Biomedical) search using the terms "malignant melanoma middle ear" from 1/1/2006 showed that there were only 3 published case reports concerning malignant melanoma in the middle ear (Park et al, 1999; Oueslati et al., 2001; Ozturk et al., 2006).

In the present study, we analysed melanocyte distribution for the first time in cholesteatoma using anti-MART-1 and anti-HMB-45 antibodies. MART-1 is a transmembrane protein that is present in normal melanocytes and malignant melanoma. Fetsch et al. evaluated MART-1, also called Melan-A, in normal tissue and in various nonpigmented neoplasms. The authors found that MART-1 stains a higher percentage of lesions than HMB-45 (Fetsch et al., 1998). MART-1 is a highly sensitive marker for intraepidermal melanocytes. In addition, there may be expression of Melan-A in keratinocytes and nonmelanocytic cells (El Shabrawi-Caelen et al., 2004). HMB-45 is an antibody directed against a premelanosome glycoprotein (Fetsch et al., 1999). Therefore, HMB-45 stains melanocytes with immature melanosome formation. Reactive or proliferating melanocytes present in inflamed adult skin, or in skin overlying certain dermal neoplasms, can also "re-express" the HMB-45-defined antigen (Bacchi et al., 1996). HMB-45 is a monoclonal antibody directed against human melanoma cells and which stains epidermal and dermal melanoma cells, the junctional components of common and dysplastic melanocytic nevi, and melanocytes in fetal skin. In addition, melanocytes in a variety of reactive conditions have been shown to label with HMB-45, as have dermal melanocytes within Spitz and dysplastic nevi. No melanocytes in normal adult epidermis or in the dermis of common nevi have stained with HMB-45 (Smoller et al., 1991). HMB-45 may correlate best with factors that stimulate melanocytic proliferation and production of melanosomes (Skelton III HG et al., 1991).

The aim of our study was to identify and determine the distribution of melanocytes in human adult primary acquired cholesteatoma and normal meatal skin. To our knowledge, this is the first study investigating the expression and distribution of melanocytes in human cholesteatoma, which may establish a new perspective to the mechanism of cholesteatoma pathogenesis.

Material and methods

All tissue specimens were obtained from patients requiring middle ear surgery. The study was approved by University of Bochum Ethics Committee, and patients were consented for skin biopsies in addition to the planned otologic procedure. 18 cholesteatoma and 10 normal auditory canal wall skin specimens were analyzed with respect to anti-HMB-45- and MART-1-expression in adult patients (ten male, eight female white Caucasian patients, mean age 52±19 years). The surgically removed specimens were immediately fixed in formalin and embedded in paraffin. All specimens were examined histopathologically with routine HE staining. All sections (4 µm) were deparaffinized and rehydrated. They underwent heating pretreatment at 70°C for 24 hours in water bath. Sections were incubated in 10 mmol citrate buffer, pH 6. The Histostain-Plus Kit (Zymed, No.85-9643) containing 10% normal non-immune goat serum was used. The samples were incubated with diluted primary antibodies [anti-HMB-45 antibody (DakoCytomation, Cat#M0634), anti-MART-1 antibody (Dako M7196 Culture supernatant 0.2 mL/mL)] at 4°C temperature overnight. After washing with tris buffered saline (TBS), the sections were incubated for 10 minutes with a biotinylated secondary antibody [Histostain-Plus Kit (Zymed, No.85-9643, San Francisco, USA)]. Streptavidin-peroxidase (Camon Laboratories, Wiesbaden, Germany) was used as an enzyme conjugate for 10 minutes at room temperature. Color development was obtained with diaminobenzidine (DAB; Camon Laboratories, Wiesbaden, Germany), yielding a brown-colored precipitate. Sections were counterstained with Meyer’s hematoxylin and mounted with aqueous mounting medium (Aquatex, Merc). The omission of the primary antibody served as a negative control. Anti-CD68 and Anti-S100, as well as Prussian blue stain were used for further characterization of pigmented cells (Dako, Carpinteria, CA, USA). Tissue specimens were examined and digitally recorded using an Olympus BX41 light microscope (Olympus America Inc., USA). Tissue specimens were planned otologic procedure. 18 cholesteatoma and 10 normal auditory canal wall skin specimens were analyzed with respect to anti-HMB-45- and MART-1-expression in adult patients (ten male, eight female white Caucasian patients, mean age 52±19 years). The surgically removed specimens were immediately fixed in formalin and embedded in paraffin. All specimens were examined histopathologically with routine HE staining. All sections (4 µm) were deparaffinized and rehydrated. They underwent heating pretreatment at 70°C for 24 hours in water bath. Sections were incubated in 10 mmol citrate buffer, pH 6. The Histostain-Plus Kit (Zymed, No.85-9643) containing 10% normal non-immune goat serum was used. The samples were incubated with diluted primary antibodies [anti-HMB-45 antibody (DakoCytomation, Cat#M0634), anti-MART-1 antibody (Dako M7196 Culture supernatant 0.2 mL/mL)] at 4°C temperature overnight. After washing with tris buffered saline (TBS), the sections were incubated for 10 minutes with a biotinylated secondary antibody [Histostain-Plus Kit (Zymed, No.85-9643, San Francisco, USA)]. Streptavidin-peroxidase (Camon Laboratories, Wiesbaden, Germany) was used as an enzyme conjugate for 10 minutes at room temperature. Color development was obtained with diaminobenzidine (DAB; Camon Laboratories, Wiesbaden, Germany), yielding a brown-colored precipitate. Sections were counterstained with Meyer’s hematoxylin and mounted with aqueous mounting medium (Aquatex, Merc). The omission of the primary antibody served as a negative control. Anti-CD68 and Anti-S100, as well as Prussian blue stain were used for further characterization of pigmented cells (Dako, Carpinteria, CA, USA). Tissue specimens were examined and digitally recorded using an Olympus BX41 light microscope (Olympus America Inc., USA). Tissue specimens were assessed in the epidermis and cholesteatoma using the automatic analyzing system (DPSOFT version 3.2, New York, USA).

The immunohistochemical evaluation was performed as follows: the assessment of the immunocytochemical staining intensity was considered as negative (no reaction), weakly positive (<5% positive cells), positive (5-75% positive cells) and strongly positive (>75% positive cells).

Results

Histiocytes and macrophages were positive for anti-CD68, but negative for S-100 in skin and cholesteatoma.
samples (Fig. 1). Anti-S100 labeled dendritic cell types like Langerhans’ cells in the skin epidermis and in higher density within cholesteatoma matrix. The Prussian blue iron stain demonstrated no positive reaction in either of the investigated groups (Fig. 2).

Anti-HMB-45 positive cells were observed predominantly adjacent to the basement membrane in normal skin (Fig. 3, Table 1). The observed melanocytes within the epidermis accounted for approximately 10% of the total cell number. Conversely, melanocytes were not observed in the cholesteatoma tissue with anti-HMB-45 in any of investigated samples (Fig. 4, table 1).

MART-1 positive cells were observed in all investigated skin samples (Fig. 5, Table 1).

Increased immunoreactivity was found within the

<table>
<thead>
<tr>
<th>antibodies</th>
<th>Normal meatal skin</th>
<th>cholesteatoma</th>
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<tbody>
<tr>
<td>Anti-HMB-45</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Anti-MART-1</td>
<td>+</td>
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++, >5% and <70% labeled cells; +, <5% labeled cells; -, 0% labeled cells.

Table 1. A survey of immunohistochemical melanocyte expression data.

Fig. 1. Numerous anti-CD68 positive macrophages in subepithelial connective cells in perimatrix of cholesteatoma. x 400

Fig. 2. Prussian blue iron stain demonstrated no positive reaction in cholesteatoma tissue. x 100

Fig. 3. Single melanocyte labeled with anti-HMB-45 antibody among the subepithelial connective cells in a regular external ear canal sample. x 400

Fig. 4. No immunoreactivity with anti-HMB-45 antibody within cholesteatoma tissue. x 200
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Discussion

Melanocytes are specialized cells that are located in the basal layer of the epidermis attached to neighboring keratinocytes (Jimbow et al., 1991). We observed brown-colored nuclei within the melanocytes of both skin and cholesteatoma. Melanocytes synthesize and transfer melanin pigments to keratinocytes. Melanosomes are vesicles that store melanin and shuttle it to the keratinocytes in specialized processes known as dendritic processes originating from a melanocyte located within the basal layer of the epidermal stratified epithelium (Zhang et al., 2004).

Melanin pigment is present in the epithelial cells as well, commonly referred to as melanogenic metaplasia. It probably appears due to transformation of epithelial cells into melanocytes (Lin and Zak, 1982).

The pathogenesis of cholesteatoma is under debate and different pathways have been suggested to lead to the same destructive lesion. It is generally accepted that the stratified squamous epithelium is derived from the epidermis of the tympanic membrane and external canal skin (Olszewska et al., 2004). Epithelial cones growing toward the underlying connective tissue exhibited high mitotic activity. The results of this study confirm a highly significant increase in the proliferation rate of cholesteatoma keratinocytes (Sudhoff et al., 1995). Park and co-authors confirmed the high number of newly formed keratinocytes in the basal and suprabasal layers, and the increased turnover rate of the basal cells. Such increased mitotic activity leads to the extensive accumulation of the keratinous mass, which induces changes in cellular differentiation and proliferation (Park et al., 1999). A study conducted by Soyer et al. in specimens of psoriasis and melanocytic skin tumors demonstrated that antigen Ki-67 is a marker of cell proliferation of the epidermis (Soyer et al., 1989). The presence of Ki-67 positive cells has been observed in the suprabasal layer and stroma of cholesteatoma in a significantly higher percentage than in normal skin by authors in the previous studies (Bujia et al., 1996).

Single melanocytes were also observed among the mesenchymal cells. As far as the epidermis is concerned, there are numerous publications in literature. They revealed the presence of melanocytes in the normal epidermis (Thong et al., 2003; Bhawan et al., 2005). Our study is the first one addressing the question of melanocyte expression in cholesteatoma. The proliferation of cutaneous melanocytes in the skin is
normal and suppressed by close physical association with epithelial cells. Numerous factors produced and released from keratinocytes may be involved in regulating the proliferation and differentiation of epidermal melanocytes (Halaban, 2000; Imokawa, 2004). The interaction between keratinocytes and melanocytes has already been described in normal human skin. Keratinocytes have been reported to stimulate the proliferation and melanogenesis in epidermis (Seiberg et al., 2000). Investigations on growth factors in cholesteatoma revealed a significantly higher expression of keratinocyte growth factor (KGF) mRNA and fibroblast growth factor (FGF) in cholesteatoma compared to normal skin, which may contribute to the hyperproliferative state in cholesteatoma. Melanogenesis and melanosome transfer from the melanocytes to neighboring keratinocytes are modulated by autocrine and paracrine factors. Among the paracrine mediators of human keratinocyte growth and differentiation are KGF and FGF. The study conducted by Cardinali et al. assessed the influence of KGF on melanosome transfer in co-cultures of keratinocyte and melanocytes.

According to that investigation, KGF is able to induce melanosome transfer on the recipient keratinocytes (Cardinali et al., 2005). It is hypothesized that such a mechanism may exist in cholesteatoma, although it should be more intensive due to a heavy immune cell infiltration. Instead of that, we observed less melanocytes in cholesteatoma epithelium compared with normal skin. The possible explanation could be the stimulation of melanocyte apoptosis by growth factors, like fibroblast growth factor-2 (FGF-2). An enhanced expression of FGF-2 and vascular endothelial growth factor (VEGF) in middle ear cholesteatoma demonstrates rapidly growing activated keratinocytes and endothelial cells. Keratinocytes of cholesteatoma are able to release angiogenic factors. Angiogenesis enables and supports the migration of keratinocytes into the middle ear (Sudhoff et al., 2000).

Invasive and hyperproliferating behavior of the cholesteatoma epithelium may be induced by a loss of growth-inhibiting signals, by an increase in growth-promoting factors, or both of these changes. It is possible that the proliferation of melanocytes is consequently controlled and responds to keratinocyte-derived signals. It seems that this is possible in two ways: 1) keratinocyte proliferation and melanocyte migration into the surrounding of keratinocytes; 2) squamous metaplasia and melanocyte metaplasia. Although there is no direct histological or experimental evidence that these mechanisms lead to cholesteatoma formation, it could be a new direction in the complicated pathogenetic pathways seen in that middle ear disease.

Conclusions

The melanocyte distribution pattern of skin was found to be different to that of cholesteatoma.

Differences concerning the morphological appearance, number and densities of melanocytes are likely to be related to the more intense neovascularization and increased level of growth factor activity in cholesteatoma. The presence of melanocytes in skin and cholesteatoma suggests a common pathogenetic origin. The occurrence of melanocytes in cholesteatoma may be due to an ingrowth controlled, as well as responding to, keratinocyte-derived signals.

Alternatively, squamous metaplasia and melanocyte metaplasia cannot be excluded.

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References


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