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Comparative cytokeratin distribution patterns in cholesteatoma epithelium

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Summary. Cytokeratins (CKs) are known as the intermediate filament proteins of epithelial origin. Their distribution in human epithelia is different according to the type of epithelium, state of growth and differentiation. We used monoclonal mouse antibodies against cytokeratins to study CK expression in the following human tissues: cholesteatoma, middle ear mucosa, glandular epithelium, and meatal ear canal epithelium. Immunohistochemical processing was performed using the labeled steptavidin peroxidase method to demonstrate the presence of CKs in cells of human epidermis. Positive reaction was obtained for CK4, CK34ßE12, CK10, CK14 in skin and cholesteatoma epithelium. However, a more extensive positive reaction with those CKs was observed in cholesteatoma epithelium. Positive immunoreactivity was seen with anti- CK19 in the glandular epithelium. Middle ear mucosa specimens revealed positive immunoreactivity with the antibodies against CK4. The expression of CK4 was definitely positive within the basal layers of the epidermis. The glandular epithelium showed no positive reaction with anti- CK4, anti-CK34ßE12, anti- CK14 and anti-CK10. Immunohistochemistry for CK18 showed no reaction in all examined tissues. Cholesteatoma is known as a proliferative disease in the middle ear which pathogenesis is not completely understood. Keratinocytes express hyperproliferation- associated CKs and after reaching the suprabasal layers they finally undergo apoptosis creating keratinous debris. Cytokeratin expression observed in the epithelium explains proliferative behavior of cholesteatoma which is associated with increased keratinocyte migration. Cytokeratins can be used as potential proliferative markers. It can also allow for searching the usefulness of inhibiting regulators in the treatment of hyperproliferative diseases.

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Introduction

Cytokeratins (CKs) have been introduced as markers of cellular proliferation. They are insoluble, low-molecular-weight proteins that form the intermediate filaments of mammalial cells (Moll et al., 1982; Hatzfeld et al., 1987; Rieger and Franke, 1988). Cytokeratins are known as the intermediate filament proteins of epithelial origin. There are about 30 polypeptides of cytokeratin (CK) expressed by different human epithelia. Cytokeratins are composed of two groups of molecules: basic and acidic. Most acidic cytokeratins coexpress with a specific basic cytokeratin to form a cytokeratin pair (Franke et al., 1981; Jorcano et al., 1984; Cooper et al., 1985; Hermann et al., 1999). Their distribution in human epithelia is different according to the type of epithelium, state of growth and differentiation. Cytokeratin expression has been widely found in various malignancies although they are also observed in other diseases that are not classified to a metastatic origin i.e. cholesteatoma. Cytokeratins 18 and 19 are usually found in simple epithelia whereas cytokeratins 5 and 14 are usually expressed by the basal cells of stratifying epithelia (Lee et al., 1994). In the pathogenesis of inflammatory diseases, cytokeratins express their role and act as messengers between cells. They act over short ranges in an autocrine or paracrine manner. They are pleotropic cell regulators, being produced not in isolation and not by a single cell type but as complex networks interregulating each other's production (Akimoto et al., 2000). The adhesion molecules control cellular interactions within the immune system and play an important role in regulating leukocyte homing (Sreeter et al., 1988; Yagita and Okumura, 1993). Changes in the expression of adhesion molecules on endothelial cells and circulating leukocytes are important factors in controlling the formation of cellular exudates

at sites of inflammation (Dustin et al., 1988). Cholesteatoma is associated with an intense inflammatory reaction with bone destruction and remodeling (Sudhoff et al., 2003; Olszewska et al., 2004). Cholesteatoma of the middle ear is a cyst-like structure composed of a keratinizing squamous epithelium matrix that contains epithelial keratin debris and a subepithelial connective tissue (Sasaki and Huang, 1994). It is characterized by invasion of the middle ear cavity by epithelium of the external auditory meatus and the tympanic membrane. The immune response is critical for the primary defense against middle ear infection (Bernstein, 1988). Cytokeratins are released by macrophages, lymphocytes, and other cells at the site of infection. They are mediators of inflammation and the immune response (Yellon et al., 1991). Cholesteatoma of the middle ear may lead to the destruction of middle and inner ear structures, causing hearing loss, vestibular dysfunction, facial paralysis and finally lethal intracranial complication (Chole, 1997). Morphologically, it is characterized by the presence of keratinizing stratified squamous epithelium accompanied by an inflammatory reaction of the subepithelial connective tissue (Hamzei et al., 2003).

In the present study, we investigated the immunoexpression of cytokeratins in different epithelial sites (cholesteatoma epithelium, middle ear mucosa, glandular epithelium, and meatal ear canal epithelium) in order to assess their exact localization in the particular layers of epithelium and their relation to hyperproliferative disease. For this study the following cytokeratins have been chosen: CK4, CK34ßE12, CK10, CK14, CK18 and CK19. We believe that cytokeratins can be used as potential proliferative markers and their expression observed in the epithelium explains proliferative behavior of cholesteatoma that is associated with increased keratinocyte migration.

Materials and methods

Strategy of selection of sections for immunohistochemical staining

Twenty five epithelium specimens from cholesteatomas in the middle ear were obtained immediately after ear surgery. Specimens of normal adult auditory meatal skin (n=10) served as controls. The middle ear mucosa and glandular epithelium came from normal samples. The glandular epithelium came from the middle ear mucosa.

The normal human tissue samples were obtained from an unaffected area of the operated ear in the same patient. Before the surgery patients were asked for the permission of taking normal samples of meatal skin and middle ear mucosa for this investigation.

The specimens were rapidly fixed in 10% formalin in the operating room from two to seven days, and then embedded in paraffin cubes. Paraffin-embedded sections were cut at a thickness of 5 mm and processed for the H&E staining in a routine way.

The indirect immunoperoxidase technique by using murine monoclonal antibodies was performed. Antigen unmasking treatment was as follows:

a) for sections examined for CK4, CK14,CK19 and CK34ßE12 antigens, the special heating treatment was performed which did not damage them [the procedure followed Liang et al., 2003]. Sections were placed in a glass staining jar filled with 0.01 mol/L citrate buffer at ph 6.0, and the jar was incubated in a 70°C water bath for 24 hours.

b) for sections examined for CK18 antigen, exposure to trypsin pretreatment was used,

c) for sections examined for CK10 antigen, none was required.

Endogenous peroxidase activity was inhibited by the preincubation of sections with 3% hydrogen peroxide for 10 minutes. Slides were then washed in TBS (Tris-Base 30.28g, NaCl 40 g, Tween 20 1.25g Aquae dest. Ad 5000 ml) three times for 2 minutes and inhibited by the Blocking solution (ZYMED, San Francisco, CA, USA). Then they were incubated for 10 minutes at room temperature. Primary antibodies for CK10, CK14, CK18, CK19 were diluted adequately in 1:200, 1:500, 1:1500, 1:100. For CK34ßE12 and CK4, primary antibodies were ready to use (adequately: Immunon™ Thermo Shandon, Pittsburgh, PA, USA and INC, Costa Mesa, CA, USA). All primary antibody reactions were incubated at 4°C overnight and then rinsed three times in TBS for 2 minutes each.

The secondary antibody used in this study was biotynylated mouse immunoglobulin (ZYMED) and incubated for 10 minutes at room temperature. Then sections were rinsed 3 times for 2 minutes in TBS. Sections were then incubated with enzyme conjugate (HRP-Streptavidin, ZYMED) at room temperature for 10 minutes. The reaction was stopped by rinsing the sections 3 times in TBS for 2 minutes each time.

For visualization of the reaction, a substrate of the Histostain®-Plus DAB kit (ZYMED) was prepared for each section which was then incubated for 3-10 minutes at room temperature. The reaction was terminated by rinsing the slide with TBS. For counterstaining, slides were dipped into Mayer's hematoxylin for 1 minute and in distilled water for 10 minutes, and then mounted.

Immunohistochemistry assessment has been done by two independent scientists on blind-labeled sections. The immunoreactivity was considered as negative when no reaction was observed, weakly positive when less that 5% of cells were positive, definitely positive- 5-70% of positive cells and strongly positive when more that 70% of cells were positive. To collect and digitalize data, an automatic analyzing system (KS300, Kontron Elektronik, Köhn, Germany) was used.

Results

Cytokeratins CK4, CK10, CK14, CK18, CK19 and CK34ßE12 were all immunolocalized in different tissues

i.e. the epidermis, cholesteatoma epithelium, middle ear mucosa and glandular epithelium. The intensity of staining was compared with noninflamed external meatal skin specimens.

Cytokeratin 4

Middle ear mucosa specimens revealed positive immunoreactivity with the antibodies against CD4. The expression of CK4 was definitely positive within the basal layers of the epidermis. The glandular epithelium showed no positive reaction in the immunohistochemical staining.

Cytokeratin 34ßE12

CK34ßE12 was weakly expressed in the basal layers of the epidermis of external meatal skin. The expression of examined cytokeratin was significantly stronger in the suprabasal layers (43% of the positive cells). The expression of CK34ßE12 in cholesteatoma epithelium assessed as a definitely positive reaction. Middle ear

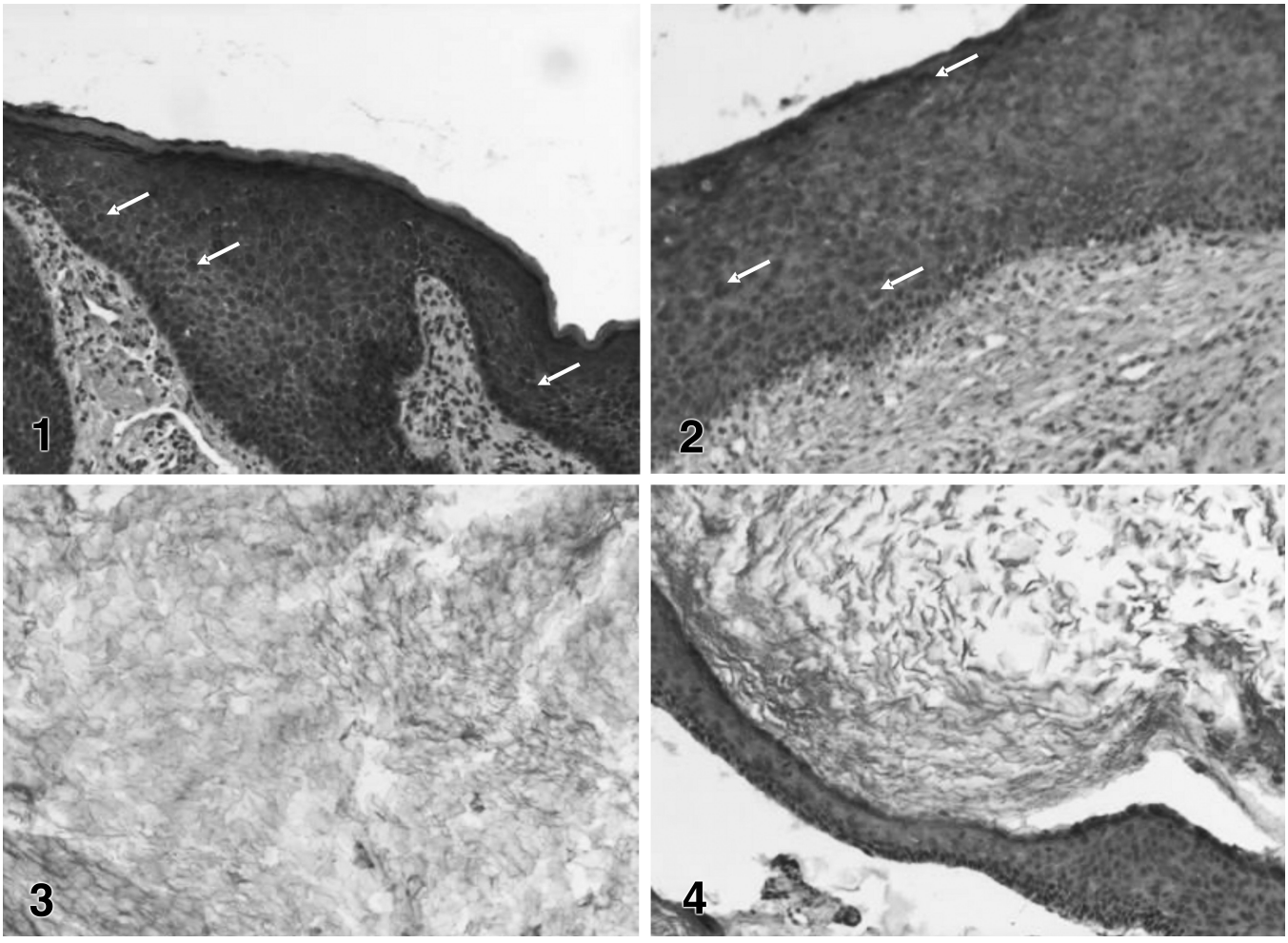

Fig. 1. CK34ßE12 is stained in the basal and suprabasal keratinocytes (arrows) of epidermis (normal meatal skin). A labeled streptavidin peroxidase method. x 200

Fig. 2. The basal and suprabasal layers of cholesteatoma epithelium show proliferating cells. CK34ßE12 staining in keratinocytes (arrows). x 200

Fig. 3. A labeled steptavidin peroxidase immunostaining for CK10 in middle ear mucosa. The immunohistochemistry shows negative reaction in the examined area. x 200

Fig. 4. The basal and suprabasal layers of cholesteatoma epithelium show weakly positive immunoreactivity with anti-CK14 in cholesteatoma epithelium. x 200

mucosa as well as glandular epithelium revealed no immunoreactivity in the whole examined area.

Cytokeratin 10

CK10 was normally localized in the suprabasal epithelial layers of the epidermis with the expression of a definitely positive reaction within the suprabasal layers and weakly positive immunoreactivity within the basal layer (less than 5% of positive cells). Neither the mucosa of the middle ear nor the glandular epithelium showed positive reaction in the observed area. The suprabasal layer of cholesteatoma epithelium revealed a definitely positive reaction (62% of positive cells) however there was no immunoreactivity observed within the basal layer of the epithelium.

Cytokeratin 14

In normal specimens, CK14 was localized in the basal layer of normal meatal epidermis with weakly positive immunoreactivity. No reaction was observed within the suprabasal layer of the epidermis. The basal and suprabasal layers of cholesteatoma epithelium showed a weakly positive reaction. Middle ear mucosa revealed a weakly positive reaction with the investigated cytokeratin. However, middle ear glandular epithelium revealed no reaction.

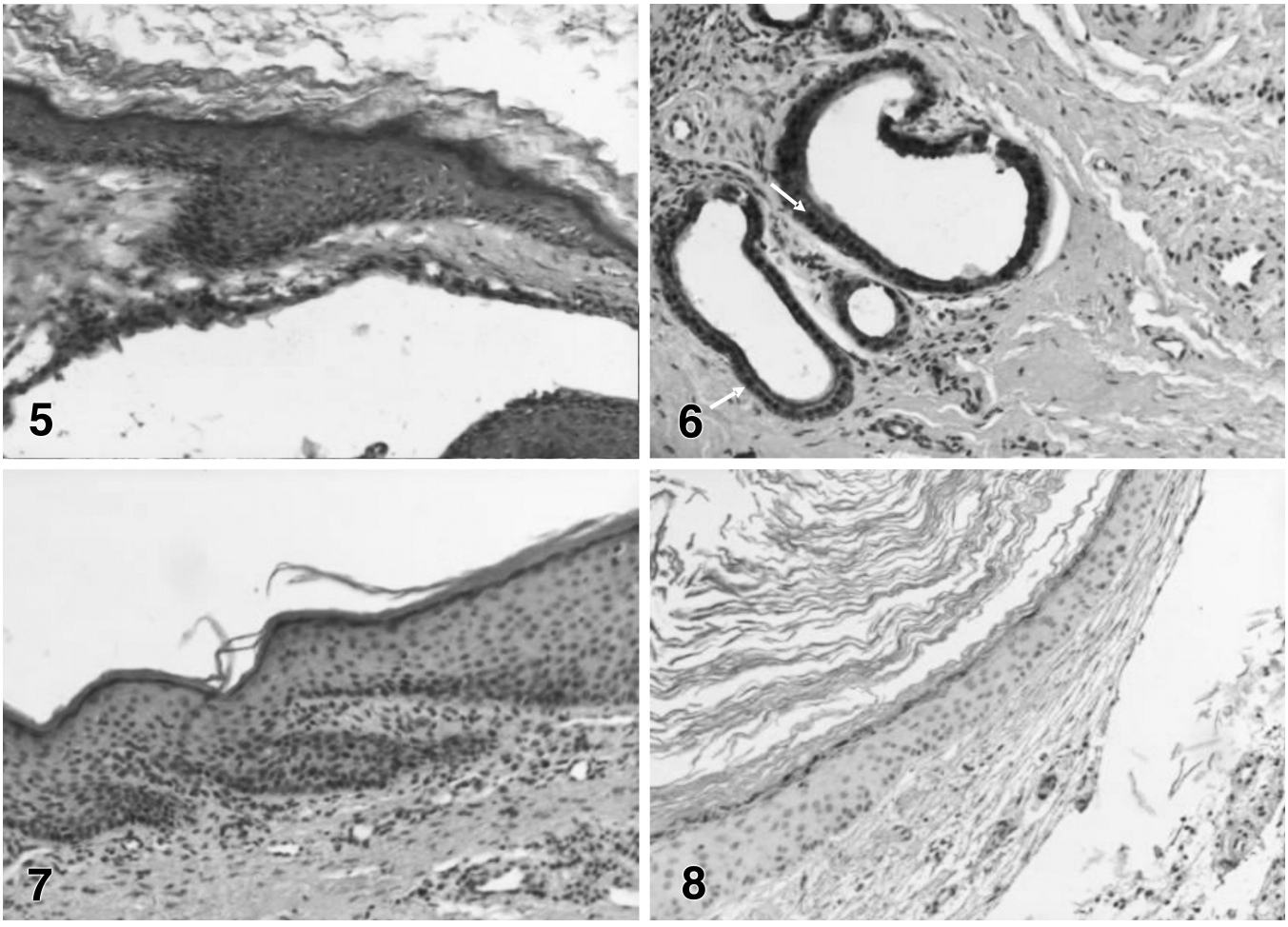

Fig. 5. Negative immunostaing of keratinocytes within cholesteatoma epithelium. The tissue section was stained with anticytokeratin antibody CK18. x 200

Fig. 6. Immunoreactivity for CK19 (arrows) in the glandular epithelium (definitely positive reaction). x 200

Fig. 7. Immunoreactivity for CK19 was analyzed by immunostaing with the anti-CK19 antibody using a labeled streptavidin peroxidase method. No positive reaction is observed within any layers of epidermis. x 200

Fig. 8. A labeled streptavidin peroxidase method with the anti-CK19. No expression observed within cholesteatoma epithelium. x 200

Table 1. A survey of immunohistochemical cytokeratin expression data.

TISSUE	LOCATION	CYTOKERATIN					
		CK4	CK10	CK14	CK18	CK19	CK34bE12
Middle ear mucosa	Suprabasal	++	-	±	-	++	-
	Basal	++	-	±	-	++	-
Middle ear glandular epithelium	Suprabasal	-	-	-	-	+	-
	Basal	-	-	-	-	+	-
Meatal epidermis	Suprabasal	++	+	-	-	-	+
	Basal	+	±	±	-	-	±
Cholesteatoma	Suprabasal	+	+	±	-	-	+
	Basal	+	-	±	-	-	±

++ > 70% labeled cells, + > 5% and < 70% labeled cells, ± < 5% labeled cells, - 0% labeled cells

Cytokeratin 18

Immunohistochemistry for CK18 showed no reaction in all examined tissues (epidermis, mucosa, glandular epithelium, and cholesteatoma epithelium).

Cytokeratin 19

No expression of CK19 was observed in any layers of the epidermis and the epithelium of cholesteatoma, however the CK reactivity in the mucosa was strongly positive (85% of positive cells) for the CK19 antibody. Cytokeratin 19 was also observed within the glandular epithelium. The reactivity in the glands was considered as a definitely positive reaction.

A survey of immunohistochemical cytokeratin data is shown in table 1.

Discussion

The pattern of CK expression correlates well with the state of keratinocyte proliferation, migration and differentiation. The keratinocytes stimulate collagenase production by fibroblasts. An epithelial-mesenchymal interaction plays a crucial role in accelerating bone destruction (Moriyama et al., 1984). One of the most striking features of cholesteatoma is the resorption of bone in the area adjacent to perimatrix. Establishing the cytokeratin pattern in different epithelia is important to the determination of proliferative capacity in several diseases. The differences among the aggressiveness of cholesteatoma growth in patients are still not completely explained. We examine cholesteatoma in contrast to the normal aural meatal skin, glandular epithelium and middle ear mucosa. The proliferative behavior of cells in normal and pathologic conditions was documented (Celis and Celis, 1985; McCawley et al., 1998; Park et al., 2001). However, the exact pathological mechanism of that process is still not completely understood. Cytokines such as: interleukin-1, epidermal growth factor, tumor necrosis factor-α and ß have been isolated in cholesteatoma tissue (Ahn et al., 1990; Yan and Huang, 1991a,b). We demonstrated that CK10, CK14 and CK34ßE12 are present in the epidermis and the epithelium of cholesteatoma. These cytokeratins react with cytokeratin intermediate filaments. CK10 and CK34ßE12 are synthesized mostly in the suprabasal layers and recognized as increased differentiation and keratinization of squamous epithelium. CK10 and CK34ßE12 are weakly expressed in the basal layers of the epidermis but significantly stronger expressed in cholesteatoma epithelium according to our study. CK4 stains basal layers of normal epidermis and appears in less differentiated keratinocytes. CK4 also shows positive reaction with middle ear mucosa. Neither the mucosa of the middle ear nor the glandular epithelium showed positive reaction in the observed area. The glandular epithelium shows positive reaction with CK19. The reactivity in the glands was considered as a definitely positive reaction. It is in agreement with (Quinlan et al., 1986; Liang et al., 2003).

Kim et al. pointed that CK expression significantly increases at the peripheral part of tympanic membrane of experimental cholesteatoma. That region is considered as the most expanding part of cholesteatoma where changes of CK expression are more pronounced. The authors concluded that CK expression patterns support the epidermal migration theory in cholesteatoma pathogenesis (Kim et al., 2002a).

Cytokeratins immunohistochemistry can aid in evaluating of hyperproliferative diseases such as cholesteatoma. Cytokeratins used in the present study have a relatively limited expression profile. Cytokeratins CK10, CK34ßE12 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. CK19 is specific for glandular epithelium and middle ear mucosa. CK4 is a marker for non-keratinizing epithelia. CK4 indicates an altered differentiation and migration of keratinocytes. According Kim et al. the patterns of CK expression correlate with the state of keratinocyte proliferation, migration, and differentiation. The authors present the concept of cytokeratin expression in

cholesteatoma as the keratinocyte differentiation into keratinizing cells. CK10 in cholesteatoma epithelium pointed to highly keratinizing process while the expression of CK4- less differentiated keratinocytes. Both CK4 and CK10 might suggest a terminal differentiation in the pathogenesis of cholesteatoma (Kim et al., 2002b).

The proliferation of keratinocytes seems to be associated with increased keratinocyte migration in certain sites within the epidermis and cholesteatoma epithelium. The results of our study also allow for searching the inhibiting regulators in the treatment of hyperproliferative diseases.

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