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Cellular signaling in human cholesteatoma

Huisman, Margaretha Aleida

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Chapter 6

Survival signalling and terminal
differentiation in cholesteatoma
epithelium.

6

MARGRIET A. HUISMAN¹, EMILE DE HEER² and JAN J. GROTE¹

*Departments of ¹Ear, Nose & Throat and ²Pathology, Leiden University Medical
Center, The Netherlands.*

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Abstract

Background: In a previous report, we have demonstrated minimal apoptosis in cholesteatoma epithelium. The phosphoinositide 3-Kinase/ Akt/Protein Kinase B (PI3K/ Akt/PKB) and the mitogen activated protein kinases (MAPK) signaling transduction pathways have been reported to protect epithelial cells against apoptosis. Both pathways have also been proven to regulate late terminal differentiation of keratinocytes. In cholesteatoma epithelium, we recently have shown MAPK activation, associated with terminal differentiation.

Objective: To investigate whether in human cholesteatoma epithelium protection against programmed cell death by means of PI3K/ Akt survival signaling is present and associated to MAPK activation and terminal differentiation.

Design: 15 samples of cholesteatoma and 15 paired control retro-auricular skin samples were immunohistochemically stained for pAkt/PKB, phosphorylated extracellular regulated kinase1/2 (pERK1/2) phosphorylated JNK/SAPK, phosphorylated p38, involucrin and filaggrin. Positive cells were counted by computer-assisted digital image analysis.

Subjects: Human acquired cholesteatoma and patient-matched retro-auricular skin were collected during surgical eradication of the cholesteatomas.

Results: The protein expressions of pAkt/PKB, pERK1/2, pp38, and involucrin in cholesteatoma epithelium were significantly increased when compared to retro-auricular skin ($p < 0.01$). Filaggrin expression was significantly decreased ($p = 0.03$). The positive correlation was confirmed between both, pERK1/2 and pp38, and involucrin ($p < 0.05$).

Conclusions: There is a strong indication that epithelial keratinocytes in cholesteatoma are protected against apoptosis. The late terminal differentiation program in cholesteatoma epithelium is disturbed.

Introduction

Cholesteatoma is a gradually expanding destructive epithelial lesion of the temporal bone. Insight into its exact pathogenesis remains incomplete. There is increasing evidence that acquired cholesteatoma behaves as a chronic wound-healing process with often an inflammatory tissue repair reaction¹. The injury is regarded as a weakening and/ or perforation of the tympanic membrane and a subsequent invasion of external ear keratinocytes into the middle ear cleft². Chronic middle ear inflammation is usually the preceding event. In general, inflammatory (oxidative) stress is a common phenomenon in cholesteatoma. Inflammatory signals induce a variability of cellular responses in which programmed cell death is a regular outcome³. In cholesteatoma epithelium, an increased apoptosis has been reported^{4,5}. However, by TUNEL assay and active Caspase 3 immunohistochemistry, we could not confirm these results⁶. We therefore hypothesize, that in cholesteatoma epithelium keratinocytes are protected against programmed cell death. The most prominent candidate process for survival of epithelial cells is the phosphoinositide 3- Kinase/ Akt/protein kinase B (PI3K/ Akt/PKB) signaling pathway^{7,8}. PI3K/ Akt/PKB is activated in cells exposed to diverse stimuli such as hormones, growth factors and integrin

ligation to extracellular matrix components. Activation of Akt/PKB by phosphorylation at position Ser 473 has been demonstrated to lead to increased keratinocyte survival⁷. The PI3K/ Akt/PKB signaling pathway however, is not the only survival pathway in epithelial cells. The extracellular regulated kinase1/2 (ERK1/2), a member of the mitogen activate protein kinases (MAPK), has also been reported to protect epithelial cells from apoptosis^{9,10}. MAPKs are a family of proteins, which are the targets for diverse extracellular stimuli that mediate a series of distinct signaling cascades. MAPK signaling is important in the regulation of a multitude of cellular functions, such as proliferation, differentiation, and apoptosis, as well as development, growth, and inflammation¹¹. Although PI3K/ Akt/PKB and MAPK pathways are seemingly independent, they appear to be both essential for epithelial survival. Interestingly, different reports mention that there is correspondence between the pathways leading to keratinocyte survival and terminal differentiation^{12,13}. Calautti et al revealed that PI3K signaling to Akt promotes keratinocytes differentiation versus death. They demonstrated that inhibition of PI3K caused a significant decrease in the amount of the late terminal differentiation markers, loricrin and filaggrin¹⁴. Efimova et al reviewed the basic role of MAPK signaling in which MAPK member p38, in concert with pERK1/2, promotes the transcription of involucrin, an early terminal differentiation marker¹⁵. In cholesteatoma epithelium, besides minimal apoptosis, we have shown ERK1/2 and p38 MAPK activation, to be associated with terminal differentiation⁶. However, to our knowledge, the expression of the communicating survival and differentiation pathway PI3K/Akt has not been investigated. We therefore questioned whether in cholesteatoma epithelium protection against programmed cell death by means of PI3K/ Akt signaling is present and if present, whether this survival signaling is associated to MAPK-activation and terminal differentiation. To investigate this, we determined the expression of the activated downstream effectors of the PI3K/ Akt and MAPK pathways resp phospho-Akt (pAkt), phospho-ERK1/2 (pERK1/2), phospho-SAPK/JNK (pSAPK/JNK) and phospho-p38 (pp38) as well as involucrin and filaggrin as terminal differentiation markers by quantitative immunohistochemistry and we correlated their expression level.

Materials and methods

Clinical and histopathological data

Acquired cholesteatoma specimens and biopsies of retro-auricular skin were obtained from fifteen patients. The samples were placed in phosphate-buffered saline immediately after the operation. The Committee of Medical Ethics (CME) of the Leiden University Medical Centre approved the protocol. The specimens were prepared for histological examination by fixation in 4% buffered formaldehyde for 20 hours followed by dehydration in ethanol and embedding in paraffin wax

Immunohistochemistry

Sections of 4 µm were taken from each tissue block. The first and the last sections were stained with hematoxylin eosin. The antibodies used were against phospho-Akt (Ser473; 1:150), phospho-ERK1/2 (Thr202/Tyr204; 1:200), phospho-SAPK/JNK

(Thr183/tyr185;1:50), phospho-p38 (Thr180/Tyr182, clone 12F8;1:50) all from Cell Signaling Technology (Beverly, MA, USA). Anti-involucrin (clone SY5; 1:10,000) and anti-filaggrin (15C10; 1:3000) were purchased from respectively Sigma-Aldrich (Saint Louis, Miss, USA) and Monosan (Uden The Netherlands). Kidney sections with diabetic nephropathy (pAkt), colon carcinoma (pERK1/2), mamma carcinoma (pp38 and pSAPK/JNK) and abdominal skin (involucrin and filaggrin) were used as positive controls. Sections from the same tissue served as negative controls, i.e. the primary antibody was omitted.

To inactivate endogenous peroxidase, the deparaffinized sections were treated with Tris-Buffered Saline (TBS) containing 0.3% hydrogen peroxide for 20 minutes. The sections were then subjected to microwave antigen retrieval in citrate buffer (0.01M, pH 6.0, 12 min.)¹⁶. To determine protein expressions an indirect immunoperoxidase method was used. Briefly, after antigen retrieval all sections were incubated with the primary antibody overnight at 4°C. After washing in TBS the sections were incubated with ChemMate Envision (anti-rabbit and anti-mouse DAKO, Glostrup, Denmark) for 30 min. at RT and developed with 3,3'-diaminobenzidin chromogen (DAB+, 1:50, 5 min. RT, DAKO, Glostrup, Denmark). The sections were counterstained with hematoxylin for 1 min.

Morphometric analysis of immunohistochemical data

The sections were analyzed using an image analysis system (Leica Microsystems Imaging Solutions Ltd. Cambridge, UK.). The microscope was a Leica DMLB with N Plan 20x0.4 objective and a Leica DC 200 digital camera. The computer-assisted system used to determine the immunohistochemical positive staining has been described elsewhere¹⁷. For each section, images from at least five different areas were stored as digitized images. For cell counting the same areas of the sections, but with various antibodies, were used. The epithelial compartment was delineated on the screen and the positive and negative cells were counted automatically. In each section > 1000 cells were counted, and the percentage of positive cells was determined. For determining involucrin- and filaggrin positivity, in 25 different locations of the epithelium the thicknesses of the DAB positive layers and the total epithelium were measured. The percentages were then calculated with regard to the total thickness.

Data analysis

Data are expressed as means $\pm$ SD. In order to compare the means of paired variables, we used the paired samples t-test. The level of significance was at $p < 0.05$. The Pearson's correlation test was used to calculate correlations. Correlation was considered significant at the 0.05 level. The SPSS10 software package (SPSS, Chicago, IL, USA) was used for the calculations.

Results

Histopathological findings

In twelve out of the fifteen cholesteatoma samples we found signs of inflammation: a locally thickened and hyperproliferative epithelium and in the connective tissue inflammatory cells and newly formed blood vessels. In two tissue samples there was insufficient connective tissue for analysis. We found no evidence of inflammation in the retro-auricular skin sections.

Immunohistochemistry

Cytoplasmic expression of pAkt in cholesteatoma epithelium was focally localized, from the lower suprabasal layers till the stratum granulosum and stratum corneum. In the retro-auricular skin, cytoplasmic pAkt was found only in some cells in the basal layers. Nuclear expression of pAkt was sometimes present in cholesteatoma as well as retro-auricular skin epithelium (Fig.1G and J). Of the pairs examined, 93 % of the cholesteatoma epithelium was more positive than its control sample. The average pAkt expression in cholesteatoma epithelium was significantly increased when compared to retro-auricular skin ($p < 0.001$). The average percentages are listed in Table 1.

In cholesteatoma and control tissue, pERK1/2 expression was mainly nuclear and localized in all layers of the epithelium (Fig.1A and D). We found all cholesteatoma epithelia more positive than its control. On average, the cholesteatoma samples had a significantly increased percentage of pERK1/2-positive cells, when compared to retro-auricular skin ($p < 0.001$). The average percentages are summarized in Table 1.

In cholesteatoma epithelium nuclear expression of pJNK/SAPK was present in all epithelial layers (Fig.1B). This was consistent with the expression pattern in retro-auricular skin (Fig.1E). We found 40% of the cholesteatoma epithelium more positive than control epithelium, which was not a significant difference. The average percentages of pJNK-positive cells in retro-auricular skin and cholesteatoma epithelium are summarized in Table 1.

The nuclear expression of pp38 was positive in all cholesteatoma suprabasal layers and sometimes also in the basal layers (Fig. 1C). This expression pattern was also observed in control skin (Fig.1F) In 80% of the pairs examined, cholesteatoma epithelium was more positive than control epithelium. When compared to retro-auricular skin, the average percentage of pp38 in cholesteatoma tissue was significantly increased ($p < 0.001$). The average percentages of pp38-positive cells in retro-auricular skin and cholesteatoma epithelium are expressed in Table 1.

Involucrin expression was prominently present in all suprabasal layers in cholesteatoma epithelium and sometimes also in the basal layers (Fig. 1H). This was different from retro-auricular skin in which the involucrin expression was mainly present in the upper suprabasal layers (Fig. 1K). We found in all of the pairs examined cholesteatoma epithelium more positive than its control. When compared to retro-auricular skin, the percentage of involucrin positivity in cholesteatoma epithelium

was significantly increased ($p < 0.001$). The average involucrin positivity is presented in Table 1.

In cholesteatoma epithelium, filaggrin expression was in general diverse, it was often absent or modestly present, but it was also focally increased and extended from the stratum granulosum to the stratum corneum (Fig. 1I). This was in contrast with the control skin, in which filaggrin was equally expressed in the stratum corneum (Fig. 1L). In 73% of the pairs examined filaggrin expression was decreased in cholesteatoma epithelium when compared to its control skin, which was a significant difference ($p = 0.003$). The average percentages are summarized in Table 1.

Tissue	pERK1/2	pJNK/ SAPK	pp38	pAkt	involucrin	filaggrin
Control skin						
mean	5.2 ± 10.3	8.1 ± 9.8	43.2 ± 0.0	20.1 ± 8.2	40.1 ± 0.5	15.7 ± 4.5
range	0.0 to 39.5	0.1 to 30.0	7.6 to 80.6	1.2 to 70.4	24.6 to 7.5	0.5 to 30.0
Chol. Ep.						
mean	20.9 ± 5.6*	10.2 ± 9.4	74.1 ± 20.6*	62.9 ± 8.4*	88.7 ± 7.4*	11.7 ± 5.4**
range	1.3 to 54.6	0.1 to 26.1	44.0 to 100.0	41.0 to 93.5	72.0 to 96.4	1.3 to 43.1

Table 1.

Percentages of pERK1/2, pJNK/SAPK, pp38, pAkt, involucrin and filaggrin-positive cells in control skin and cholesteatoma epithelium. Involucrin and filaggrin positivity is expressed as the percentage of epithelium stained when compared to the total thickness.

* $p < 0.001$; ** $p < 0.003$ versus control skin

Associations of pAkt, pERK1/2, pJNK/SAPK, pp38, involucrin and filaggrin

Using the Pearson's correlation test, a significant positive association was observed between involucrin- and pERK1/2 expression ($p = 0.05$) and between the expressions of involucrin and pp38 ($p = 0.04$).

Discussion

In this study we demonstrated that in cholesteatoma epithelium active Akt is present. When compared to control skin the expression of activated Akt was significantly increased. Filaggrin expression on the contrary, when compared to control skin, was virtually absent, although some focal strong positivity was observed in individual cases. As previously demonstrated, ERK1/2 and p38 MAPK activation was increased and associated with involucrin expression.

Phosphorylated Akt has been extensively reviewed as a promoter of cell survival^{7,18}. It operates via multiple mechanisms such as maintaining mitochondrial integrity and the phosphorylation and thus inactivation of the pro-apoptotic protein BAD^{7,18}. The increased presence of activated Akt in cholesteatoma epithelium may therefore indicate protection against programmed cell death, which is in line with our previous findings. PI3K/ Akt signaling also has been demonstrated to be involved in late

terminal differentiation, however, we were not able to establish an association between pAkt and filaggrin¹⁴. Instead, the overall filaggrin expression was decreased when compared to control skin. This is in contrast with a previous report in which filaggrin expression in cholesteatoma epithelium has been found to be increased¹⁹. Different types, differential heterogeneity of cholesteatoma tissue or the stage of development may account for this discrepancy. Alternatively, in different keratotic skin diseases such as psoriasis and granular parakeratosis an absence or reduction in the amount of filaggrin has been reported²⁰⁻²². In one of these reports it has been mentioned that augmented apoptosis explains the diminished presence of late terminal differentiation markers in psoriasis²¹. In that study the TUNEL assay has been used but in another study DNA analysis revealed that psoriatic keratinocytes have intact DNA²³. Metze et al suggested that abnormalities of cell surface adhesion structures might account for the dysregulation of the cornified envelope components²². This has recently been supported in the report of Calautti, in which evidence has been provided that differentiation-specific activation of the PI3K pathway requires the cadherin-catenin adhesion complexes¹⁴. Thus, protection against apoptosis occurs by activated Akt, but initiation of late terminal differentiation needs an additional component: cell adhesion. In their analysis of cholesteatoma tissue integrity Naim et al found, contrary to normal skin, that beta-catenin was diminished or absent in cholesteatoma suprabasal layers²⁴. Their proposal that matrix metalloproteases (MMPs) play a prominent role in this process may hold true, because MMP activity has been frequently reported in cholesteatoma²⁵.

MAPK activation has been reported to generate early terminal differentiation by promoting involucrin transcription¹⁵. Interestingly, sustained ERK1/2 MAPK activation is required for anchorage-independent survival of epithelial cells¹⁰.

The increased presence of the activated ERK1/2, p38 MAPK and involucrin and their associations in cholesteatoma epithelium indicate, that the MAPK early terminal differentiation pathway is active. Increased presence of activated Akt, however, is in the greater part of cholesteatoma epithelium not followed by filaggrin expression. It appears therefore that the process of late terminal differentiation is abrogated and that keratinocytes are arrested in an early terminal differentiation stage. This phenomenon has also been described for other keratotic skin diseases²². The defect in the production of filaggrin may result in a failure to degrade keratohyalin granules²². This may explain the conspicuous granular appearance of the cholesteatoma cornified layer¹. In summary, we established increased activated Akt in cholesteatoma epithelium, which was not associated to MAPK-mediated terminal differentiation. In the reduction of filaggrin expression in cholesteatoma epithelium we found a disturbed late terminal differentiation program consistent with other keratotic skin diseases. Our future research will concern cytokine/ growth factor involvement in cholesteatoma terminal differentiation.

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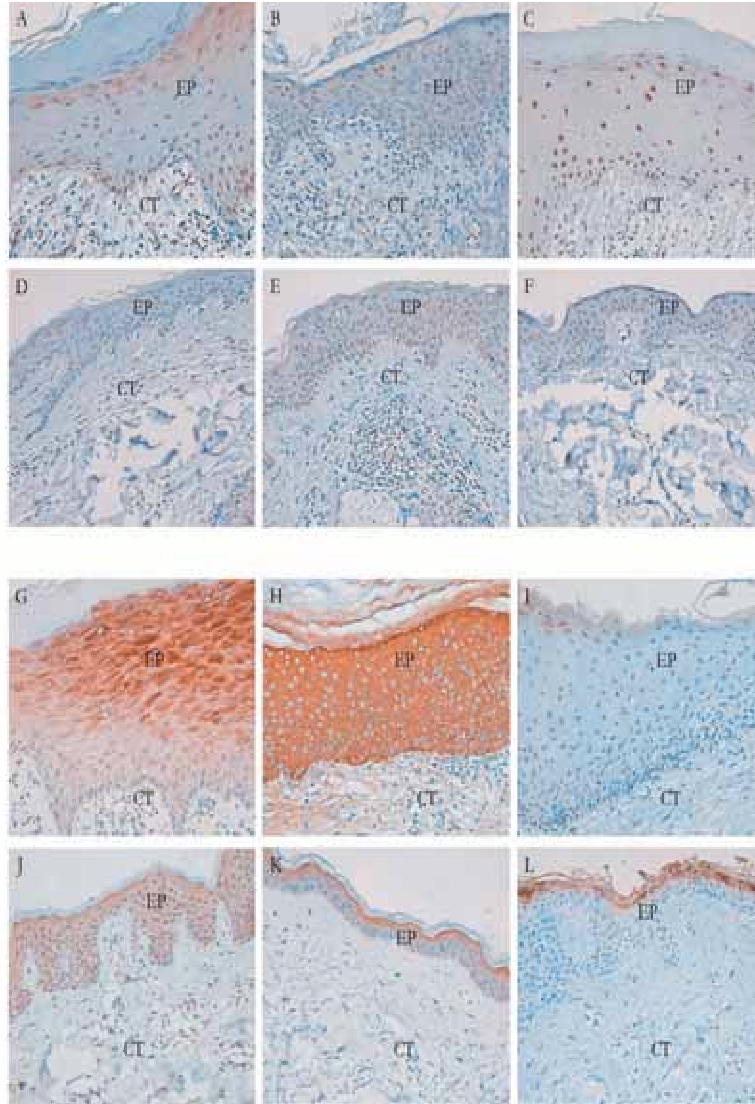


Figure 1. Immunohistochemical analysis/ staining of paraffin sections of human cholesteatoma epithelium (A,B,C,G,H,I) and retro-auricular skin(D,E,F,J,K,L). Localization of p ERK1/2(A,D), pJNK/SAPK(B,E), pp38(C,F), pAkt(G,J), involucrin(H,K) and filaggrin(I,L) in Original magnification: x 200.

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