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Cellular signaling in human cholesteatoma

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Chapter 5

Terminal differentiation and mitogen-activated protein kinase signaling in human cholesteatoma epithelium.

5

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Abstract

Objectives:

To investigate whether - in cholesteatoma epithelium - terminal differentiation, resulting in high involucrin expression, is associated with mitogen-activated protein kinase (MAPK) signaling.

Background:

Alterations in specific signal transduction pathways may explain abnormal differentiation of the keratinocytes in cholesteatoma. Signaling pathways used by eukaryotic cells to transduce extra cellular signals into cellular responses converge on activated mitogen-activated protein kinases, mainly extracellular signal-regulated kinase, c-Jun NH2-terminal kinase and p38.

Materials and Methods:

Tissue samples were taken from 16 patients with acquired cholesteatoma. Histologic examination showed that 12 of the 16 cholesteatoma were inflamed. Immunohistochemical methods were used to determine expressions of involucrin and the activated form of p38 (pp38), extracellular signal-regulated kinase and c-Jun NH2-terminal kinase proteins. The results obtained from cholesteatoma tissue were compared with paired control samples from retro-auricular skin

Results:

We demonstrated increased levels of involucrin and increased levels of the activated forms of p38 and ERK1/2 in cholesteatoma epithelium when compared with control samples. No abnormality was found in the activation and expression of JNK1/2. A positive correlation was found between pp38, pERK1/2, and involucrin expression ($p<0.05$).

Conclusions:

Our results demonstrate that signaling via the mitogen-activated protein kinases ERK1/2 and p38 is increased in cholesteatoma epithelium when compared with control skin. The correlations between involucrin- and phosphorylated pERK1/2 expression and between involucrin - and phosphorylated p38 expression indicates that terminal differentiation in cholesteatoma epithelium proceeds via activation of these mitogen-activated protein kinase signaling pathways. We discussed whether this increased mitogen-activated protein kinase-driven terminal differentiation is probably part of a keratinocyte survival program or caused by an inflammation-induced cellular stress response.

Introduction

In normal human epidermis, the process of terminal differentiation is tightly controlled and takes place in its suprabasal layers. One of the main proteins involved in this process is involucrin, the precursor of the keratinocyte cornified envelope, which is selectively expressed in the upper suprabasal layers. The final product, a thin layer of cornified envelopes, is continually shed from the surface of the epidermis. This requires a regular supply of *de novo* differentiated cells. In cholesteatoma, this process differs from the normal situation[1]. Terminal differentiation of cholesteatoma epithelium is aberrant and is characterized by involucrin expression in all suprabasal cell layers, which results in massive

accumulation of keratin debris[1]. Cells respond to extracellular signals by transmitting intracellular instructions to coordinate fundamental cellular responses. The mitogen-activated protein kinase (MAPK) cascades are among the best characterized of these intracellular signaling pathways. In mammalian cells, these MAPK cascades consist of three distinct kinase routings downstream. These pathways include Ras/ Raf/ MEK1/ ERK1/2 (MAPK ERK kinase1/ extracellular-regulated kinase1/2), Ras/ JNK/ SAPK (c Jun N-terminal kinases/ stress-activated protein kinases) and the Ras/ p38 MAPKs. Activated and, thus, phosphorylated MAPKs translocate to the nucleus, where they activate transcription factors and target genes (Fig.1)[2]. Efimova et al. has demonstrated that among the MAPKs, p38 is probably the most important kinase, which is required for human involucrin promoter activation[3]. Parallel pathways, however, might also play a role, such as the Ras/ ERK1/2-p38[4] and Ras/ ERK1/2-JNK[5] pathways in psoriasis, indicating that different signaling pathways may be active in keratinocytes. For a better understanding of the molecular basis of cholesteatomal terminal differentiation, we studied which MAPK signaling pathways are involved in this process. For this purpose, we determined the expressions of activated ERK1/2, JNK, p38, and the corresponding expression of involucrin. The results obtained from cholesteatoma tissue were compared with paired control samples from retroauricular skin.

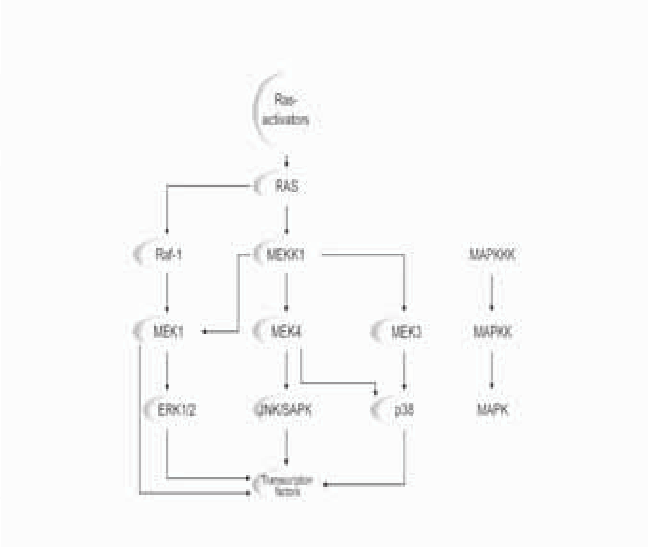

Figure 1. The classic MAPK cascade consists of three sequental intracellular activation steps and is initiated when the first member, MAPKKK, is activated. MAPKKK activates MAPKK. Subsequently, MAPKK activates a MAPK. There are three MAPK pathways downstream including Ras/ Raf/ MEK1/ ERK, Ras/ MEKK1/ MEK4/ JNK/ SAPK and the Ras/ MEKK1/ MEK3/ p38 MAP kinases. Crosstalk between different MAPK pathways might occur.

Materials and methods

Clinical and histopathological data

Cholesteatoma specimens and biopsy specimens of retro-auricular skin were obtained from 16 patients. The samples were placed in phosphate-buffered saline immediately after the operation. The Committee of Medical Ethics 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 and eosin. The sections were immunostained with antibodies, respectively, against phosphorylated-ERK1/2 (pERK1/2)(Thr202/Tyr204), phosphorylated-JNK/SAPK (pJNK/SAPK)(Thr183/Tyr185), phosphorylated-p38 (pp38)(Thr180/Tyr182) (clone 12F8) and involucrin (clone SY5). Anti-pERK1/2, anti-pJNK/SAPK and anti-pp38 were obtained from Cell Signaling Technology (Beverly, MA, U.S.A). Anti-involucrin was purchased from Sigma-Aldrich (Saint Louis, Mo, U.S.A). The dilutions used were as follows: anti-pERK1/2, 1:200; anti-JNK/SAPK, 1: 50; anti-pp38, 1:50; and anti-involucrin, 1: 10,000. Sections from the same tissue served as negative controls (i.e. the primary antibody was omitted). Sections from colon carcinoma (pERK1/2), mammacarcinoma (pp38 and pJNK/SAPK) and abdominal skin (involucrin) were used as positive controls. The expressions of pERK1/2, pJNK/SAPK, pp38 and involucrin were determined using indirect immunoperoxidase methods. After deparaffination, all sections were treated with tris buffered saline (TBS) containing 0.3% hydrogen peroxide for 10 minutes to inactivate endogenous peroxidase. The sections assigned for pERK1/2, pJNK/SAPK and pp38, were subjected to microwave antigen retrieval in boiling citrate buffer (0.01mol/L, pH 6.0, for 10 minutes)[6]. These sections were, before immunolabeling, pretreated with TBS/0.1% Tween for 5 minutes. The sections were then incubated with the primary antibodies against pERK1/2, pJNK/SAPK, and pp38, in TBS/0.1% Tween, overnight at 4°C. After washing in TBS, the sections were incubated with ChemMate Envision (anti-rabbit; DAKO, Glostrup, Denmark) for 30 minutes at room temperature (RT) and developed with 3,3'-diaminobenzidin (DAB+) chromogen (1:50, for 5 minutes RT; DAKO). The sections for anti-involucrin immunolabeling were incubated overnight at RT. They were then washed with TBS, incubated with the appropriate biotinylated secondary antibody for 30 minutes at RT, washed with TBS, and subsequently incubated with peroxidase-conjugated streptavidin (DAKO) at RT for 30 minutes. They were then treated with DAB chromogen containing 0.002% H_2O_2 for 15 min. All sections were counterstained with hematoxylin for 1 minute.

Morphometric analysis of immunohistochemical data

For each of the immunohistochemical markers studied, DAB-positive cells were counted using an image analysis system (Leica Microsystems Imaging Solutions Ltd. Cambridge, U.K.). The microscope was a Leica DMLB with N Plan 20 x 0.4 objective and a Leica DC 200 digital camera. The computer-assisted system used to determine the immunohistochemical positive staining has been described

elsewhere[7]. 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 more than 1,000 cells were counted, and the percentage of positive cells was calculated. For determining involucrin-positivity, in each section, on 25 different locations of the epithelium the thicknesses of the involucrin-positive layer and the total epithelium were measured. The percentage of the involucrin-positive layer was then calculated with regard to the total thickness.

Data analysis and statistics

Data are expressed as means ± SD. Because of a non-Gaussian distribution of parameters, we used the non-parametric Wilcoxon signed-rank test for comparison of the differences between protein expressions in cholesteatoma and retro-auricular skin. The level of significance was at $p < 0.05$. The Spearman's rank correlation test was used to calculate correlations. Correlation was considered significant at the 0.05 level. The SPSS Version 10 software package (SPSS, Inc.,Chicago, IL, U.S.A.) was used for the calculations.

Results

Histopathological findings

In 12 of the 16 cholesteatoma samples, we found signs of inflammation: inflammatory cells, newly formed blood vessels in the connective tissue and locally thickened epithelium. In two tissue samples, there was insufficient connective tissue for analysis. In the retroauricular skin sections, there was no evidence of inflammation.

Expression of pErk1/2, pJNK/SAPK, pp38 and Involucrin

The expression of pERK1/2 in cholesteatoma epithelium was mainly nuclear and localized in all layers of the cholesteatoma epithelium (Fig. 2A). This observation was consistent with the expression of pERK1/2 in retroauricular skin. On average, the cholesteatoma samples had a significantly increased percentage of pERK1/2-positive cells, when compared with retroauricular skin ($p=0.001$). Cholesteatoma epithelium was more positive than control epithelium in 94% of the pairs examined. The average percentages of pErk1/2-positive cells in retroauricular skin and cholesteatoma epithelium are summarized in Table 1. In cholesteatoma epithelium nuclear expression of pJNK/SAPK was present in all epithelial layers (Fig. 2B). This was consistent with the expression pattern in retroauricular skin. We found 44% of the cholesteatoma epithelium more positive than control epithelium, which was not a significant difference. The average percentages of pJNK-positive cells in retroauricular skin and cholesteatoma epithelium are summarized in Table 1. In cholesteatoma, the nuclear expression of pp38 was positive in all suprabasal layers. In some cholesteatoma, pp38 expression was also present in the basal epithelial layers (Fig. 2C).Cholesteatoma epithelium was more positive than control epithelium in 75% of the pairs examined. When compared to retroauricular skin, the average percentage of pp38 was significantly increased in cholesteatoma tissue ($p=0.003$). The average percentages of pp38-positive cells in retroauricular skin and

cholesteatoma epithelium are summarized in Table 1. Involucrin expression was prominently present in all suprabasal layers in cholesteatoma epithelium and sometimes also in the basal layers (Fig 2D). This was in contrast with retroauricular skin in which the involucrin expression was mainly present in the upper suprabasal layers. In 100% of the cases, cholesteatoma epithelium was more positive than control epithelium of the pairs examined. Compared with 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.

Tissue	pERK1/2	pJNK/SAPK	pp38	Involucrin[a]
Retroauricular skin				
Mean	5.1 ± 10.0	7.6 ± 9.7	43.2 ± 19.3	39.1 ± 10.9
Range	0.1-39.5	0.1-30.0	7.6-80.6	24.7-67.5
Cholesteatoma epithelium				
Mean	19.6 ± 15.9	10.3 ± 9.1	70.9 ± 23.5	86.7 ± 10.7
Range	0.2-54.6	0.1-26.1	24.0-108.8	57-96.4

Table 1. Percentages of pERK1/2-, pJNK/SAPK- and pp38-positive cells in control skin and cholesteatoma epithelium.
[a]Involucrin positivity is expressed as the percentage of epithelium stained when compared to the total thickness.

Associations of pErk1/2, pJNK/SAPK, pp38 and involucrin with inflammation

The epithelial expressions of pERK1/2, pJNK/SAPK, pp38, and involucrin of all cholesteatoma samples showed large intra- and interindividual variations. Protein expressions were within the limits of this variance when comparing inflamed cholesteatoma samples with those in which we found no signs of inflammation. For this reason, no correlation between protein expression and inflammation could be established.

Associations of pErk1/2, pJNK/SAPK, pp38 and involucrin

Using the Spearman's rank correlation test, a significant positive association was observed between involucrin and pERK1/2 expression ($p= 0.02$) and between the expression of involucrin and pp38 ($p=0.02$). Even after control of basal protein expression level per patient, these correlations persist. We did not find any correlation between the other proteins.

Discussion

In the current study, we report that phosphorylated ERK1/2 and phosphorylated p38 are prominently present in cholesteatoma epithelium. We found that the presence of pERK1/2 and pp38 was positively associated with the expression of involucrin. Activated JNK expression did not differ from that in normal skin. In cholesteatoma epithelium MAPK signaling with relation to involucrin expression

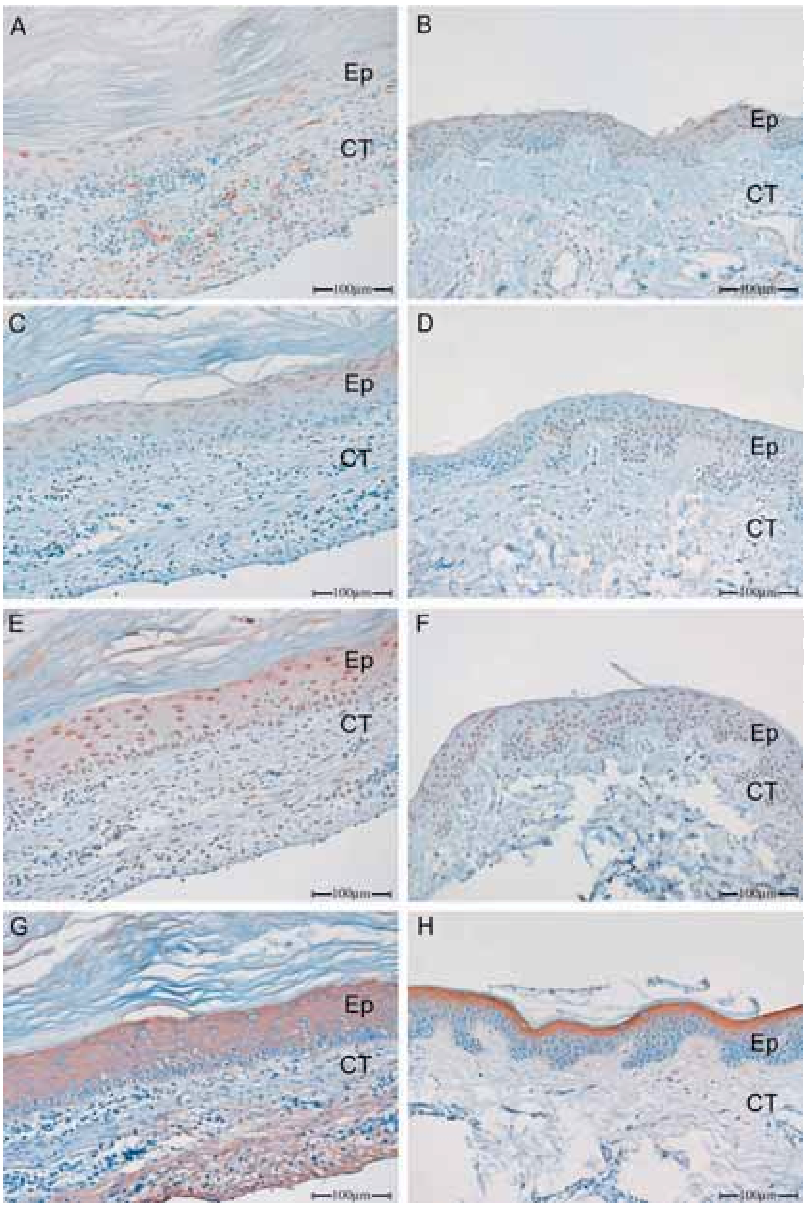

Figure 2. Immunohistochemical localization of pERK1/2, pJNK/SAPK, pp38, and involucrin in paraffin sections of human cholesteatoma epithelium and paired retro-auricular skin; (A and B) pERK1/2-positive cells are expressed in all epithelial layers; (C and D) pJNK/SAPK-positive cells are expressed in all layers of the epithelium; (D and E) pp38-positive cells are mainly expressed in all suprabasal layers, but sometimes in cholesteatoma epithelium also in the basal layers;(F and G) involucrin in cholesteatoma epithelium, positive cells are mainly expressed in suprabasal layers but also in basal layers, and in retroauricular skin involucrin-positive cells are mainly expressed in the upper suprabasal layers. Original magnification: x200. Ep, epithelium; CT, Connective Tissue

has, to our knowledge, never been reported before.
It has been demonstrated in vitro that different agents such as calcium, vitamin A, protein kinase C activators, cytokines and antioxidants, can regulate involucrin gene expression[3,8]. Efimova et al. reviewed the basic role of MAPK cascades in these involucrin promoter-activating mechanisms[3,9]. These articles contribute to the understanding regarding the mechanisms that regulate keratinocyte differentiation. In keratinocytes however, when different cytokines and growth factors are present, MAPK signaling may even cause opposing effects on involucrin transcription[10]. In cholesteatoma numerous different cytokines and growth factors have been demonstrated[1]. In our experiments, we found as a net result an increased involucrin expression, which has been demonstrated previously[11]. We also found involucrin expression associated with both an increased pERK1/2 and an increased p38 activation. This result is in contrast with previous articles in which p38 signaling was mentioned to be the most important involucrin regulatory mechanism in keratinocytes [3]. We also could not confirm the finding of Efimova et al. concerning the p38-related synchronous reduction in ERK1/2 activity[9]. There may be different explanations for this. Differences between keratinocytes in vitro versus in vivo analysis, as well as differential cytokine and growth factor triggering, may account for variations in MAPK signaling[12]. In agreement with these arguments and with our report is that, in psoriatic skin lesions, a similar MAPK activation pattern, normal pJNK expression, and increased pERK1/2 and pp38 expression, have been demonstrated[4]. Interestingly, it has been reported that, in psoriasis, involucrin expression is increased[13].
Various conditions may therefore lead to parallel pERK1/2 and p38 pathway activation. In cholesteatoma, growth factors and cytokines may activate different receptors. Growth factor receptor activation initiates the MAPK/ERK1/2 signaling pathway, whereas proinflammatory cytokines such as IL-1 and tumor necrosis factor-α regulate p38 signaling[4]. In cholesteatoma epithelium, the general cellular stress response may then be a parallel ERK1/2 and p38 signaling and subsequent augmented involucrin upregulation.
However, there may be another reason for differential ERK1/2 and p38 activation: keratinocytes that lose their contact with the epidermal basal membrane rapidly differentiate and keratinize[14]. The increased differentiation and subsequent keratinization in cholesteatoma epithelium shows remarkable similarity with this process. In general, however, when epidermal cells lose their contact with the extracellular matrix they rapidly become apoptotic, a process called anoikis[14]. Protection of epithelial cells against anoikis is associated with and requires sustained ERK1/2 MAPK phosphorylation[15]. It has also been demonstrated that inhibition of ERK signaling leads to apoptosis of keratinocytes[16]. This survival mechanism is of importance in tissue repair processing for migrating keratinocytes at the leading edge of a cutaneous wound.
This anchorage-independent survival of keratinocytes might also arise in cholesteatoma in which an aberrant and disrupted basal membrane has been demonstrated[17]. In addition, cholesteatoma keratinocytes are known to be migrative[18]. Anchorage-independent survival of cholesteatoma keratinocytes is also

in line with our previous findings demonstrating sustained active ERK1/2 expression and minimal apoptosis[19,20].

We therefore postulate that, in cholesteatoma epithelium, terminal differentiation is mediated by both, pERK1/2 and p38 MAPK activation. Whether this is part of an inflammation- induced cellular stress response and/ or part of a keratinocyte survival program during wound healing needs to be investigated. Future research will be focused on the role of different cytokines involved in cholesteatoma hyperdifferentiation.

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