Cellular signaling in human cholesteatoma

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Chapter 8

Summary and General Discussion

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To investigate cellular pathways in human cholesteatoma we made use of immunohistochemistry. We have chosen this technique for several reasons:

1. Cellular signaling pathways are post transcriptional processes, which means that the activation of such a pathway is not due to an increase in the production of a certain protein, but is due to whether or not a certain protein is activated or deactivated.
2. In cholesteatoma, epithelium and stroma are entangled (Fig. 1), which makes it virtually impossible to separate e.g., by proteolytic cleavage, epithelium from stroma and to analyze their different cells by RNA, or DNA analysis.
3. In skin, the cells from the basal layer have different protein expression patterns from those in the suprabasal layers and in the stratum corneum.
4. In this study it is essential to investigate at which location different proteins are present and activated.

In **chapter 1**, cholesteatoma is described from a general clinical, morphological, biological and molecular point of view. Briefly, different hypotheses of cholesteatoma genesis are discussed. Arguments to distinguish cholesteatoma benign character from a malignancy are considered from different points of view. In this chapter, features of cholesteatoma wound healing and inflammation processes and the complexity of its protein signaling are indicated.

A detailed description of the different cellular pathways and proteins investigated in this thesis is presented in **chapter 2**. This includes 3 downstream pathways in MAPkinase signaling-, the pAkt- and TGFb signaling pathways. Moreover, activation of p53 and its influence on apoptotic processes and the complicated role of $p21^{cip1/waf1}$ in cell cycle control is reviewed.

In **chapter 3**, differences in proliferation, cell cycle arrest and apoptosis between cholesteatoma and control skin were determined. Our results indicated that in cholesteatoma epithelium an increased expression of Ki-67, a marker of cell proliferation, is accompanied by an increase in the p53- as well as the cell cycle arrest protein $p21^{cip1/waf1}$. We also demonstrated a significant positive correlation between p53 and $p21^{cip1/waf1}$. Apoptosis is very low in the cholesteatoma epithelium. This was established by the active caspase 3 staining and the TUNEL assay. In immunohistochemistry it is of the utmost importance to use adequate controls. This has also turned out to be true for the TUNEL assay, for false positive TUNEL staining was an important problem to overcome in the first stages of this study. Of importance is that, in different reports appropriate controls were not used and pictures with apoptotic cells were not shown, which makes these studies not

reliable[1,2]. It is of importance that an apoptotic morphology and a positive apoptotic staining both must be present to be allowed to call a cell apoptotic, unfortunately, this is not always the reported[3,4].
The results in cholesteatoma epithelium indicate that the increased proliferation is not compensated by apoptosis, but might be associated with cell cycle arrest.

The results in **chapter 4** show that in human cholesteatoma epithelium the Ras/ Raf/ ERK1/2 MAPK pathway is involved in p53-dependent increased expression of $p21^{cip1/waf1}$. This was confirmed by the correlated expression of p53 and $p21^{cip1/waf1}$ and that of $p21^{cip1/waf1}$ and active ERK1/2. Interestingly, ERK1/2 activation has been associated with both stimulation and inhibition of cell proliferation. The magnitude and the duration of ERK1/2 activation determines whether the cellular response is proliferation or cell cycle arrest. ERK1/2-mediated induction of proliferation requires a transient, low ERK1/2 activation while induction of $p21^{cip1/waf1}$ expression requires a strong and sustained ERK1/2 signaling. In cholesteatoma epithelium, these differential processes were visualised in the basal and suprabasal layers. In the basal cell layers, i.e. the proliferating compartment, only pERK1/2 was expressed. In the suprabasal layers, when pERK1/2 expression was sustained, the cells were also found positive for the cell cycle arrest protein $p21^{cip1/waf1}$. Prolonged MAPK-induced cell cycle inhibition causes accumulation of epithelial cells, which are in G^0-arrest[5]. We concluded therefore that in human cholesteatoma, prevalent cell cycle arrest might contribute to epithelial hyperplasia.

Alterations in specific signal transduction pathways may explain abnormal differentiation of the keratinocytes in cholesteatoma.
In **chapter 5** the correlation between terminal differentiation and signaling via the MAPKs has been investigated. We found that the presence of pERK1/2 and pp38 was positively associated with the expression of involucrin. Activated JNK expression appeared not to be involved in this process. This indicates that terminal differentiation in cholesteatoma epithelium proceeds via activation of pERK1/2 and p38 MAPK signaling pathways. Our results were in contrast with previous articles in which p38 signaling is mentioned to be the most important involucrin regulatory mechanism in keratinocytes[6]. We also could not confirm the finding of Efimova et al concerning the pp38-related synchronous reduction in ERK1/2 activity[7]. In this chapter we argued that growth factor receptor activation and other pro- inflammatory responses may regulate a parallel ERK1/2 and p38 signaling and subsequent augmented involucrin up regulation. Another reason for autonomous activation of ERK1/2 may be an anchorage-independent survival of cholesteatomal keratinocytes. The cholesteatoma basal membrane has been demonstrated to be aberrant and disrupted[8]. Protection of epithelial cells against loss of anchorage is associated with and requires sustained ERK1/2 MAPK phosphorylation[9]. The assumption that cholesteatoma keratinocytes are subjected to anchorage-independent survival is in line with our previously demonstrated sustained active ERK1/2 expression and minimal apoptosis[10,11]. Remarkably, this survival mechanism is of importance in tissue repair processing and for migrating

keratinocytes at the leading edge of a cutaneous wound. The arguments of parallel signaling due to inflammation- induced cellular stress response or keratinocyte survival program during wound healing may positively hold true for cholesteatoma tissue (Efimova, pers comm.)

In **chapter 6**, the protein expression of pAkt/PKB, was found to be significantly increased when compared to control skin. This is concomitant with our previous reports in which we demonstrated minimal apoptosis in cholesteatoma epithelium. The late terminal differentiation marker filaggrin was found to be significantly decreased and although PI3K/ Akt signaling has been demonstrated to be involved in late terminal differentiation, we were not able to establish an association between pAkt and filaggrin[12]. We found the terminal differentiation marker involucrin significantly increased, but we were also not able to establish a correlation between pAkt and involucrin. In psoriasis , however, the same differentiation profile has been reported: an augmented involucrin expression and an absence or reduction in the amount of filaggrin[13,14]. It has been suggested that abnormalities of cell surface adhesion structures might account for the dysregulation of the cornified envelope components[15]. This has recently been supported by the report of Calautti, in which evidence has been provided that differentiation-specific activation of the PI3K pathway requires the cadherin-catenin adhesion complexes[12]. 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 layers[16]. This indicates that, in line with our previous reports, decreased or absent cellular contact in cholesteatoma epithelilum may be the cause of both increased involucrin and decreased filaggrin expression.

TGFβ is thought to be a key factor involved in wound healing and the objective of **chapter 7** was to investigate, whether TGFβ is activated in human cholesteatoma.

In the epithelium, we found concordant expressions of TGFβ and p-Smad2, but a decreased Smad7 expression when compared to its control. The correlations between TGFβ, pSmad2 and Smad7 may indicate that Smad activation and inhibition are still operational. Nevertheless, in spite of the decreased Smad7 expression these processes do not lead to a significant pSmad2 up regulation. It has been reported that besides inhibiting Smad2 activation, Smad7 appears to play a critical role in mediating apoptosis by activation of the JNK signaling pathway. The decreased Smad7- and our previously demonstrated decreased JNK expression in cholesteatoma epithelium may therefore represent protection against apoptosis. In this chapter we also compared, in a pilot study, the results of this study with the results of our study of MAPkinase signalling, and we found correlations between TGFβ, pSmad2 and pERK1/2 ($p=0.02$ and 0.03). We hypothesized that in cholesteatoma epithelium default levels of TGFβ may become effective because of co-operative signaling, leading to augmented transcription of different genes. One of these genes may be $p21^{cip1/waf1}$, which we previously found to be increased and

related to pERK1/2 expression[10]. This may be a mechanism of fine-tuning of TGFβ signaling, which indicates that protein signaling may be augmented, but not out of control.

In cholesteatoma stroma, TGFβ and pSmad2 were increased, while Smad7 expression was equal when compared to control skin. EDA-FN accumulation in cholesteatoma stroma was excessive whereas no EDA-FN expression was not detectable in control skin. In this chapter it is argued that the increased TGFβ, pSmad2 and EDA fibronectin expressions may be cellular responses to persistent inflammation. There may be several reasons for such a hypothesis: 1) the cholesteatoma particle can be considered to be a foreign body, a *corpus alienum*, which can induce a recurrent inflammatory reaction in the middle ear[17]. 2) inflammation is recurrent because in most cholesteatoma, biofilms are present which can release endotoxins[18]. Endotoxins can reinitiate inflammation because they can trigger many cells to produce cytokines. 3) inflammation will be self perpetuating because of lack of clearance in the enclosed cavity of the middle ear. There may also be a combination of these factors, or all may play a role in cholesteatoma chronicity. In this chapter we indicate that cholesteatoma is a process of chronic wound healing in which TGFβ signaling and a persistent inflammation appears to be contributing factors to its chronicity.

General Discussion

In cholesteatoma epithelium, increased expressions of those proteins were found, which are involved in proliferation (Ki-67), cell cycle arrest (p53 and $p21^{cip1/waf1}$), early expression of the terminal differentiation protein involucrin and survival (pAkt). Moreover, increased activation of MAPK- and its association with TGFβ cellular signaling cascades were demonstrated. The proteins of which the expressions were decreased were those concerning late terminal differentiation (filaggrin) and apoptosis (active caspase 3). We found different correlations between the various investigated proteins. We therefore assume that these correlations can be placed in the context of interconnected signaling processes. It appears that these connections in both MAPK- and TGFβ signaling, support cell cycle arrest, early terminal differentiation and survival (Fig.1) which are hallmarks of wound healing. This suggests that cholesteatoma epithelium behaves like a wound healing process in which the keratinocytes are migrative. Many biological characteristics of cholesteatoma epithelial cells can be explained with this concept. Increased proliferation, which is shown by the increased Ki-67 proportion in cholesteatoma epithelium, is not compensated by increased apoptosis. This can explain hyperplasia, which is a common phenomenon in cholesteatoma. Hypertrophy is also frequently present and may be caused by cell cycle arrest but also by differentiation, for it has been demonstrated that these two processes are known to induce cellular volume[19,20]. Moreover, it has been reported that keratinocytes in a wound healing process are hypertrophic, (which is also due to cell cycle arrest and aberrant differentiation)[21]. Aberrant differentiation, the early and increased expression of involucrin, but also the decrease in filaggrin expression may be due to loss of adhesion[12,15]. This loss of adhesion may be caused by an interrupted and

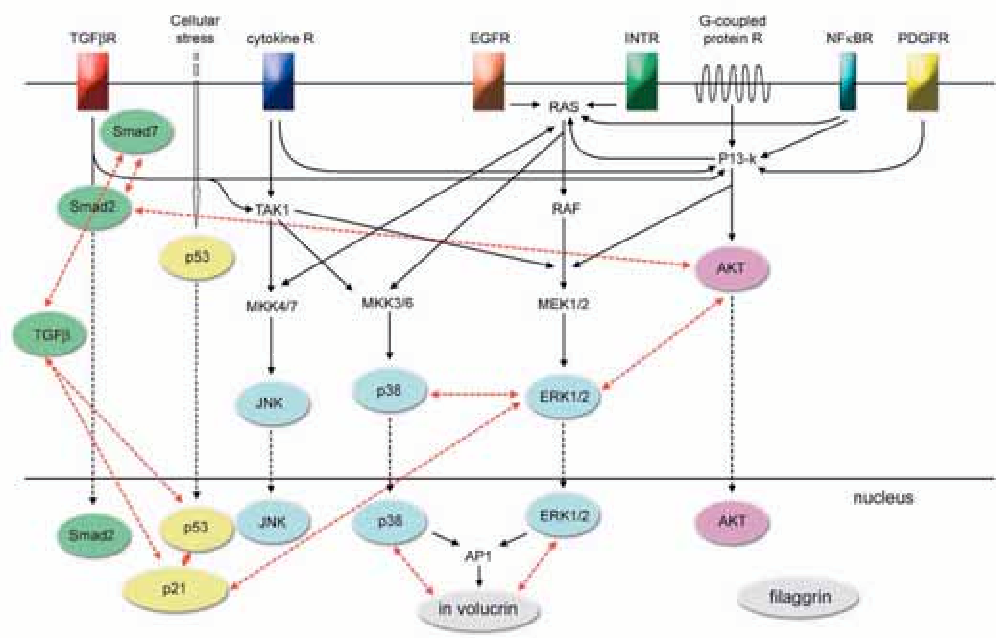

Figure 1. The pathways investigated in this thesis. The red lines represent significant correlations.

altered basal membrane and/or a decreased α catenin expression, which previously have been reported in cholesteatoma epithelium[8,16]. Moreover, this lack of adhesion is visible, because in different reports cholesteatoma pictures reveal a widening of intercellular space, the latter is also reported to be a phenomenon in wound healing[21]. The decrease of filaggrin can also explain another biological characteristic of cholesteatoma epithelium: that of parakeratosis. When late terminal differentiation is not initiated, the cells will retain their nuclei and the tissue becomes parakeratotic. Interestingly, early terminal differentiation, demonstrated by increased involucrin expression will not cease proliferation, for involucrin-positive cells are still capable to continue DNA synthesis[19].

The increased TGFβ and pSmad2 expressions in cholesteatoma stroma and the increased EDA-Fibronectin also point to a chronic wound healing process. In cholesteatoma, this process is clearly visible by the accumulation of EDA-fibronectin. Although it is chronic in cholesteatoma, we suppose that it is still part of a normal process of wound healing for EDA-fibronectin is essential in appropriate self limited wound healing[22]. Moreover, in normal wound healing, the final signal is that of the disappearance of inflammatory cells. It has been reported that this occurs by apoptosis. In chapter 3, although there was minimal apoptosis in cholesteatoma epithelium, we found many apoptotic cells in the stroma. Cholesteatoma recurrence can also be explained, for it has been reported that, after injury, cells can be reactivated even years after the first event[23].

In conclusion, we hypothesize that in cholesteatoma cellular signaling occurs along the lines of normal, but chronic, wound healing.

Further research

We recommend research into pharmacological interventions aimed at control of inflammation. Because application of antibiotics has proven to be not very successful probably because of the presence of biofilms- we believe that anti microbial peptides may be an adequate novel therapy.

To get more insight in cholesteatoma genesis we suggest to study the influence of external stimuli, such as keratin particles and endotoxins, on the protein expression and protein signaling profile in the advancing front of meatal epidermis and middle ear epithelium.

Retraction pockets are considered to be a pre-stage in cholesteatoma formation, although the development to a cholesteatoma is tentative. Of interest is therefore to compare the protein expression and protein signaling profile of retraction pocket tissue to that of cholesteatoma.

We further recommend to study whether biofilms play a role in cholesteatoma development and progress. This should be done by detection of both, bacterial colonies and their glycocalix layer, in retraction pockets and different types of cholesteatoma.

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