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Cellular signaling in human cholesteatoma

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Chapter 1

General Introduction

General clinical, morphological, biological and molecular aspects of cholesteatoma.

Clinical aspects

Cholesteatoma is a benign, gradually expanding destructive epithelial lesion of the temporal bone. Several hypotheses for the pathogenesis of human cholesteatoma have been proposed of which the most important are[1]:

- The congenital hypothesis: cholesteatoma originates from embryological ectoderm remnants in the petrous bone. This implies that cholesteatoma develop behind an intact tympanic membrane in patients without a history of aural infections.
- The metaplastic hypothesis: metaplastic changes of differentiated middle ear epithelium lead to the formation of a cornified cholesteatoma epithelium.
- Epidermal hypotheses: cholesteatoma is considered to be an intrusion of epithelium from the existing epidermal lining of the tympanic membrane or external auditory canal (ME) into the middle ear cleft, forming a pathological collision between keratinocytes and mucosa. This ME may invade into the middle ear by 1) invagination of the tympanic membrane (retraction hypothesis), 2) ingrowth over the edges of a tympanic membrane perforation (migration hypothesis) and 3) medial proliferation of the basal cells through an intact tympanic membrane (proliferation hypothesis). These epidermal hypotheses suppose a considerable migratory capacity of the cells of the external ear canal. In cholesteatoma genesis, a combination of these epidermal hypotheses seems plausible. This has indeed been proposed for the retraction- and proliferation hypotheses[2].

In this thesis acquired cholesteatoma will be investigated. The genesis of acquired cholesteatoma is based on the epidermal hypothesis. Acquired cholesteatoma will usually occur in combination with a chronic middle ear inflammation or infection. Clinical sequela may include destruction of the middle ear ossicles and other structures. When untreated, there is a risk of labyrinth involvement, which may result in vertigo and sensorineural hearing loss. Facial nerve dysfunction and intracranial injury, although rarely seen today, are serious complications[3]. Early detection of cholesteatoma is important but complicated, because the early symptoms are difficult to distinguish from chronic otitis without cholesteatoma. High-resolution computed tomography and magnetic resonance imaging may facilitate pre-operative identification of cholesteatoma, although surgical exploration remains the most effective way[3,4].

Histomorphological aspects

The epithelial compartment

The epithelium of cholesteatoma exhibits generally exhibits a heterogeneous thickness, with a majority of hypertrophic areas, adjacent to normal ones (Fig1A). The hypertrophic area is at least 3-5 times thicker than normal retro-auricular skin. This increased thickness is often not only due to the hypertrophic character of the epidermis but also to an increased number of cell layers. Focal

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hyperproliferation is present but not restricted to the hypertrophic layers. In the hypertrophic layers a modification of keratinocyte morphology is often observed. Different keratinocytes exhibit a rounded shape with hypertrophic cytoplasm and a round nucleus. There are also keratinocytes with a spindle shape which are oriented towards the stratum corneum with elongated cytoplasm and an oval nucleus. The diameter of the hypertrophic cells is about twice the diameter of normal cells. The hypertrophic areas often show a significant widening of the intercellular space, which suggests alterations in the network of intercellular junction proteins. In the non-hypertrophic areas abnormally small keratinocytes are often present, with a polygonal shape and similar to that observed in the basal layer of the normal retro- auricular epidermis (Fig.1B). The cholesteatoma epithelium has parakeratotic features, which is defined by the presence of nucleated cells in the stratum corneum. Hyperkeratinization is a common phenomenon in cholesteatoma tissue. There is a generalized inflammatory reaction with infiltration of different types of inflammatory cells into the epithelial compartment. Clusters of polymorphonuclear granulocytes (PMNs) and macrophages are present in areas adjacent to the stratum corneum

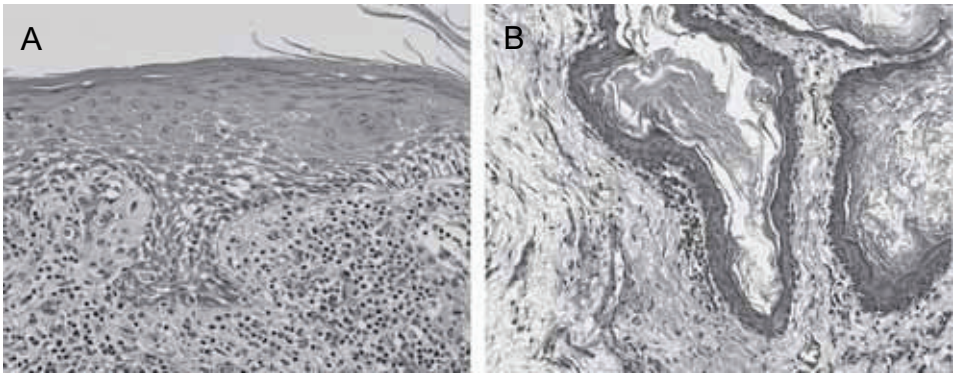

Fig.1 HE staining of a cholesteatoma. Original magnification: 200 x. Figure 1A represents a hypertrophic area with round and spindle cells. Figure 1B represents a non-hypertrophic area with very small cells.

The subepithelial compartment

Basal membrane

Cholesteatoma basal membrane differs from that of normal skin. It is often disrupted in areas where inflammation is present. Immunohistochemical investigation reveals aberrant collagen 4 and laminin expression[5]. At the ultrastructural level, protrusions, duplications, thickening and disruptions of the lamina densa of the basement membrane were observed[5].

The dermis

Epithelial papillary outgrowth is a common phenomenon. The dermis is hyalinized and shows disorganized supporting fibres such as collagens and elastin. Vascularization is two-fold when compared to normal skin[6]. Inflammation is often prominently present with abundant inflammatory cells including T-cells,

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macrophages, lymphocytes, mast cells and PMNs.

Biological aspects.

Is cholesteatoma a skin disease?

The presence of keratinising stratified squamous epithelium within the middle ear cleft has led to the assumption that cholesteatoma epithelium may be classified as a skin disease. Its parakeratotic aspect may subclassify it into the group of skin diseases such as psoriasis, dermatitis, pityriasis lichenoides, or precancerous and malignant squamous lesions[7].

Is cholesteatoma a malignancy?

It has been suggested that several morphological aspects of human cholesteatoma resemble those in pre-malignant and malignant skin diseases[8]. These aspects include: increased proliferation, atypical differentiation and chromosomal abberations. However, cholesteatoma is not a malignancy because it is not invasive and metastases have never been demonstrated. We determined the expressions of proliferation and differentiation markers of cholesteatoma and compared these with the results of other studies of cholesteatoma, malignant, pre-malignant and benign skin diseases[9-40]. We focussed on the immunohistochemical detection of the proliferation markers Ki-67 and PCNA, the suppressor gene p53 and the marker of differentiation involucrin. The results are shown in Table 1.

		Ki67 refs	PCNA refs	P53 refs	Involucrin refs
malignant	Cutaneous squamous cell carcinoma	9,10	11	12	13
	Squamous cell carcinoma	14,15	15,16	15	14
pre-	Actinic keratosis	15,17,18	15,17	15,17	19
	Bowen's disease	14,15,17	15,17	15,17,20	14
	Keratoacanthoma	15	15	15	30
non-	Treatment resistant atopic dermatitis	21,22	16	23	24
	Psoriasis vulgaris	14,25,26	16,25	25,26	14
	Verruca vulgaris	15	15,26	15,27,28	29,30
	Cholesteatoma	31-38	35,38	31,32,34-37	39,40
	Normal skin	14,18,26	18	14-18	14

positivity: 0-10% | 10-25% | 25-50% | >50%

Table 1. represents differential expression of the proliferation markers (Ki67, PCNA), p53 and a terminal differentiation marker (involucrin). The numbers refer to different immunohistochemical studies of malignant-, pre-malignant-, benign skin diseases, cholesteatoma and normal skin.

This table shows the tendency of malignant skin diseases to be hyperproliferative. Benign skin diseases often show increased differentiation[41]. When compared with normal skin, differentiation of cholesteatoma epithelium is increased but this should *de facto* be considered as evidence in favor of the benign character of the disease. It has been argued that proliferation in cholesteatoma epithelium is increased[6]. Compared with all skin diseases including benign tumours, however, the average proliferation rate is not increased. Albino *et al.*, who found only a marginally statistically significant difference in proliferation between cholesteatoma and retro-auricular skin[8], has previously discussed this. Investigation of the (increased)

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proliferative rate of cholesteatoma keratinocytes in children led to the speculation that high cholesteatomal proliferation might be considered as an indication for aggressive (i.e. fast growing) clinical behavior[42,43]. This view is not supported by other studies, which showed that clinically less aggressive cholesteatomas also have a high proliferation rate[44]. The induction of proliferative cells in suprabasal layers of the cholesteatoma epidermis might imply a potential idiopathic response to external stimuli in the form of cytokines released by infiltrating inflammatory cells.
Ki-67 is expressed throughout all phases in the cell cycle and PCNA in the S-phase but, interestingly, in cholesteatoma epithelium PCNA expression levels are higher than those of Ki-67. It has been demonstrated that PCNA is not only associated with delta DNA polymerase but also with mismatch repair genes[45]. We therefore hypothesize that in cholesteatoma, as a consequence of a possible DNA-damaging effect of inflammatory stress, the expression of PCNA could be higher than that of Ki-67.
In cholesteatoma Albino *et al.* have demonstrated normal diploid DNA contents. However, other studies have reported chromosomal aberrations, such as chromosome 8 aneuploidy and chromosome 7 triploidy[46,47]. In these studies, fluorescence *in situ* hybridization (FISH) techniques have been used. It is of note that chronic inflammatory stress, which is a common phenomenon in cholesteatoma epithelium, can also induce chromosomal aneuploidy or triploidy. Kinne *et al.*, using the same techniques, have described similar chromosomal aberrations for chromosome 7 and 8 in chronic rheumatoid arthritis[48]. Although in cholesteatoma no clonality studies have been done, we believe that cholesteatoma does not show inherent genetic instability, but that the reported chromosomal aberrations are more likely to be caused by chronic inflammatory stress.

Is cholesteatoma a defective wound healing- or an inflammatory process, or both?
Pressure-induced invaginations, morphological changes of the tympanic membrane (TM) or even perforation of the TM result in enough damage to induce wound-healing processes[8]. It has also been suggested that the juxtapositioning of two different epithelia, epidermis and middle ear epithelium, might be regarded as a persisting epidermal defect[1].

Woundhealing in cholesteatoma
The different stages of epithelial wound healing are inflammation, proliferation and demonstrated to be present (Table 2)[35,49-70]. Inflammation is illustrated by the recruitment and activation of different inflammatory cells in the subepithelial compartment[6,8]. The proliferative phase of cholesteatoma is illustrated by focal hyperproliferative epithelial growth centres[6]. Migration of the newly formed tissue to the injured site is a characteristic of remodelling. The migratory character of keratinocytes in cholesteatoma epithelium has been reported[71] and the increased presence of the αV integrin subunit in the epithelial/subepithelial interface may indicate the formation of new anchoring contacts necessary for keratinocyte motiliy[72]. Furthermore, it has been shown that cholesteatoma fibroblasts have a

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highly migrative phenotype[73]. Although features of remodelling are present in cholesteatoma, it is considered to be defective because it remains in the inflammatory phase[8].

Recently, the presence of biofilms in cholesteatoma has been demonstrated[74].

Stage of wound healing	Involved cytokines of growth factors	References
The initiator of activation:	IL- 1	49-52
Maintenance of activation:	TNF-, including upregulation of: amphiregulin, TGF -, add. IL -1, IL -1 receptor antagonist, epidermal growthfactor receptor (EGFR), EGF, KGF and ICAM	35,50,53-59
The activated phenotype responsible for additional signalling:	growth factors and cytokines including TGF, IL -3, IL-6, IL-8, G-CSF, GM-CSF and M-CSF. cell signaling processes: RAF/ ERK1/2 MAPK pathway, Ras -c-jun	35,60-64
The contractile cell, migration:	IFN-, MMPs	IFN$^{\gamma}$R:65,66-68
Extracellular matrix deposition; inhibition of cell proliferation= back to basics	TGF-, fibronectin and collagen.	35,57,62,69

Table 2. Represents different stages of epithelial wound healing according to Freedberg (70) and the relevant literature concerning cholesteatoma pathogenesis.

Biofilms are colonies of quiescent bacteria in a hydrated matrix of polysaccharides. In these biofilms the bacteria are protected against noxious micro-environmental conditions as well as high concentrations of antibiotics. Although encapsulated, bacteria can be released from the biofilm and converted into the planktonic and thus infective form. The presence of biofilms in cholesteatoma may be responsible for the chronic inflammation, caused by either the released planktonic bacteria or by the continuous released endotoxins[74] such as lipopolysaccharide (LPS). Adherence of bacteria to epithelial surfaces can induce cellular signaling and cytokine upregulation[75]. Endotoxins are able to stimulate the keratinocytes of the middle ear epithelium to cytokine production[76], which may result in recurrent inflammation. However, this is not always the default course of events because not every patient reacts to the same degree to endotoxins. Innate or acquired immunological factors may account for this individual variation[77]. When cytokines and growth factors from inflammatory cells and/or endotoxins are present they may induce metaplastic changes of the epithelium[78]. This is in accordance with the metaplastic hypothesis proposing metaplastic changes of the differentiated middle ear epithelium. In contrast, to the metaplastic hypothesis however, cholesteatoma also presents without earlier inflammation notwithstanding the fact that it is associated with inflammation.

Whether cholesteatoma is an inflammation or a wound, why does it not heal?
Many factors can impair healing, such as systemic and local factors[79]. Systemic

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factors may be very diverse, such as malnutrition, advanced age and diabetes. To our knowledge, it has not been proven that cholesteatoma do not heal due to systemic reasons. Local factors, which delay or prevent healing, include the presence of foreign bodies, tissue maceration, ischaemia and infection. Besides infection, which is a known phenomenon in cholesteatoma pathology, it is appealing to consider a foreign body as an inhibiting factor for wound healing. Cholesteatoma, which is a keratinized particle encapsulated in the middle ear, might be regarded as a *corpus alienum.* An immunological reaction is obvious and inflammation may be the consequence[80,81].

Of interest is also a report in which it has been demonstrated that wound fibroblasts generate a brisk TNF response to stimulation with LPS, while under the same conditions, normal dermal fibroblasts did not secrete any measurable amounts of TNF[82]. In cholesteatoma, the increased presence of LPS may therefore contribute to disordered wound healing[83].

In addition to systemic and local factors that impair healing, an imbalance between proteolytic enzymes and their inhibitors, or a reduction in tissue growth factors, seem to be of particular importance in chronic wounds. An imbalance between proteinases and their inhibitors may induce excessive proteinase activity, which can result in a chronic wound. Moreover, it has been suggested that growth factors can be depleted by proteases, which may also result in non-healing[84]. In cholesteatoma different reports describe the increased presence of growth factors and proteases but their degree of activity or the presence of their inhibitors, has hardly been investigated and needs to be further explored.

Molecular aspects

In cholesteatoma, the result of the chronic inflammatory process is the presence of a plethora of inflammatory cytokines and growth factors, expressed by inflammatory cells and keratinocytes. The understanding of wound-healing mechanisms has progressed considerably in recent years[85,86]. However, many questions remain, such as the considerable crosstalking in the system. Most wound signals control more than one cell activity but cell activity may also be a response to differential triggering[87]. Moreover, it is certain that growth factor and matrix signals are not the only relevant influences. Changes of gap-junctional connections between keratinocytes at the healing margin[88] may coordinate cell proliferation and migration. Mechanical signals such as cell stretching or altered tensions at the wound-site may prove to be important alternative factors in wound healing. The presence of many inflammatory signaling proteins in the more or less enclosed area of the middle ear may result in an altered or confused signal transduction within the cholesteatoma epithelial- and sub epithelial cells. To our knowledge, studies on cellular signaling pathways in cholesteatoma have not been published. The aim of this thesis is to explore the main transduction signaling pathways in cholesteatoma. Because of the complexity of the system, this study is mainly focussed on MAPK-, Akt- and TGF-β- signaling pathways in cholesteatoma keratinocytes and the TGF-β-signaling in the stroma. The proteins that are involved in these signaling pathways will be discussed in the next chapters.

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Aim and outline of this thesis

The main objective of this thesis is to investigate those protein signaling pathways in human cholesteatoma which may be involved in different aspects of cholesteatoma pathogenesis, such as hyperproliferation, aberrant differentiation and extra-cellular matrix deposition.

Aim of the study

The major objective of this study is to investigate cellular signaling pathways and the expression of different proteins in human cholesteatoma in order to answer the following questions:

1. Is increased proliferation in cholesteatoma compensated by increased apoptosis?
2. What are the signaling pathways that influence the proliferative activity of the keratinocytes?
3. What is the mechanism behind increased differentiation?
4. Which are the main processes leading to extra-cellular matrix alterations?
5. Are extra-cellular matrix alterations associated with aberrant epithelial characteristics? (Is there crosstalk between these?)
6. Can different pathogenic features of cholesteatoma be explained?

Content of the thesis

In this thesis we studied the signaling pathways in human cholesteatoma epithelium, which are involved in cellular proliferation, terminal differentiation, cell cycle arrest and apoptosis. We also investigated to which extent TGF-ß1, as the key factor involved in wound healing, is involved in both cholesteatoma epithelial and stromal cellular signaling.

Chapter 1 describes cholesteatoma from a general clinical, morphological and biological point of view.

In **chapter 2** the most important proteins involved in proliferation (Ki-67, PCNA), differentiation (involucrin) and cell cycle arrest (p53, $p21^{cip1/waf1}$) as well as the mechanism of apoptosis and the role of active caspase 3 are reviewed. In this chapter also the phenomenon cellular signaling is introduced including MAPK, pAKT and TGF-ß signaling pathways.

Chapter 3 concerns the study of the expression level of different proteins involved in proliferation, cell cycle arrest and apoptosis and their association.

Chapter 4 provides evidence for an association of the expression of $p21^{cip1/waf1}$ as a marker of cell cycle arrest and MAPK signaling.

In **chapter 5** we investigated the involvement of MAPK signaling in terminal differentiation.

Terminal differentiation of cholesteatoma epithelial cells as a survival mechanism is presented in **chapter 6**.

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Chapter 7 describes TGF-ß bioactivation in cholesteatoma epithelium as well as stroma.
The general discussion and summary are presented in **chapter 8**.

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