Cellular signaling in human cholesteatoma

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Voor mijn ouders
Voor mijn kinderen





Contents

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

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

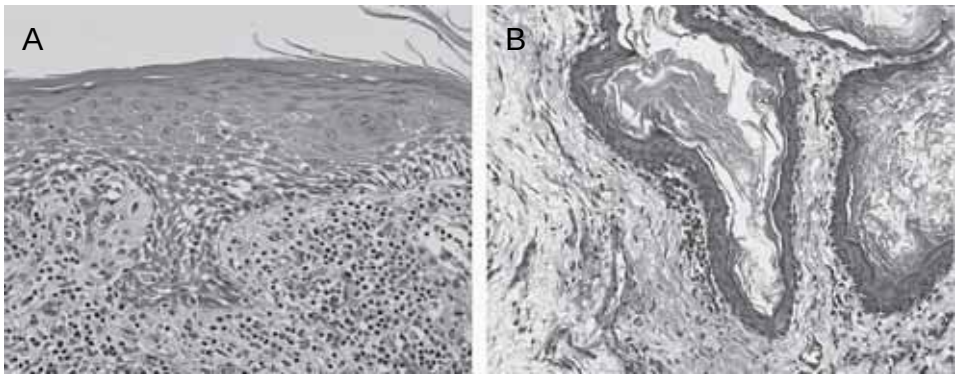

Fig.1 HE staining of a cholesteatoma. Original magnification: 200 x. Figure 1A represents a hypertrophic area with round and spindle cells. Figure 1B represents a non-hypertrophic area with very small cells.

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,

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)

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

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^{7}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

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.

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**.

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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Chapter 2

2

Molecular pathways in human cholesteatoma

A comprehensive review of signaling pathways investigated in this thesis.

Cellular signaling by cytokines, growth factors and peptide hormones is generally mediated through membrane-bound receptors, whereas smaller and more lipophilic signaling molecules such as steroid hormones and some vitamins often signal through nuclear receptors. General features of cytokines and growth factors are pleiotropism and redundancy. Pleiotropism refers to the ability to act on different cell types, which reflects that different cell types may have the same type of receptors on their membrane surface. Redundancy refers to the property of multiple cytokines and growth factors having the same functional effect, which can be explained by the fact that the same receptor or receptor type can bind different cytokines or growth factors[1].

This overview will concentrate on signal transduction pathways and proteins that regulate the balance between keratinocyte cell proliferation, survival, apoptosis and differentiation, with a particular emphasis on the role of the mitogen-activated protein kinase-(MAPK), PI3Kinase/ Akt-, TGFß-signaling and the p53 and $p21^{cip1/waf1}$ protein.

MAPK signaling

The classic MAPkinase cascade consists of three sequental intracellular activation steps and is initiated upon ligand binding with the appropriate receptor. The three MAPK routes are initiated by different stimuli through different receptors but the architecture of the signaling cascades is in general similar. After ligand binding usually a set of adaptors such as sonic hedgehog (Shc) and growth factor receptor-bound protein 2 (GRB2) bind and recruit the guanine nucleotide exchange factor (GEF) SOS to the plasma membrane in proximity to Ras to exchange the GDP for GTP on Ras proteins (Ras, Rac, Rho), which in turn activate the first member of the cascade: a MAPKK kinase (MAPKKK or MEKK) (Fig.1)[2]. A MAPKKK is a serine/ threonine kinase that phosphorylates and activates MAPK kinases (MAPKK or MEK).

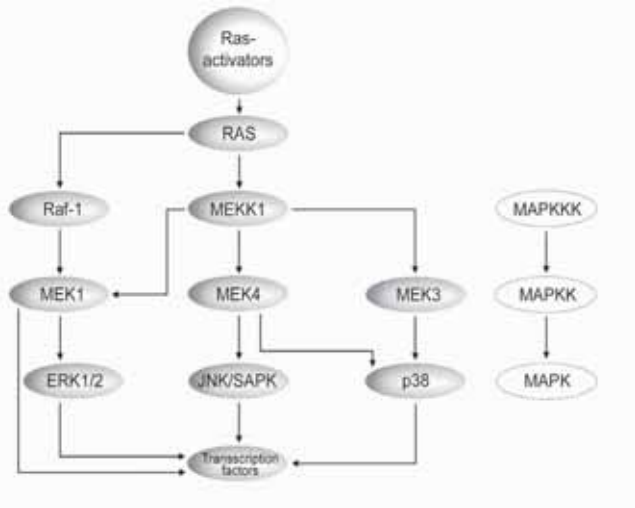

Figure 1.
The classic MAPKinase cascade consists of three intracellular activation steps and is initiated when the first member, MAPKKK, is activated. MAPKKK activates MAPKK, subsequently, MAPKK activates a MAPK. There are three MAPK pathways downstream including Ras/ Raf/ MEK1/ ERK, Ras/ MEKK/ JNK/ SAPK and the Ras/ MEKK1/ p38 MAPKinases. Crosstalk between different MAPK pathways may occur.

Subsequently, MAPKK activates a MAPK by dual phosphorylation on adjacent threonine and tyrosine residues. All three MAPK families are activated by dual phosphorylation on both adjacent threonine and tyrosine residues separated by a single amino acid, forming a tripeptide sequence. The second amino acid for MAPKs for extracellular regulated kinase (ERK) is glutamate (Thr- Glu- Tyr), for the p38 family Glycine (Thr- Gly- Tyr) and for the c-Jun N terminal-kinase (JNK) family Proline (Thr- Pro- Tyr)[3].
Several studies describe extensive crosstalk between these cascades in which a particular kinase in one pathway affects a kinase activity in another pathway. This illustrates that MAPKs are a highly interdependent regulatory network in which the cellular outcome is likely to be dependent on the balance between regulatory inputs. Full specificity is ensured through docking interactions by kinases that recognize a distinct site on their substrates. E.g., the JNK docking region of c-Jun recognizes JNK and determines thereby its specific phosporylation at ser63 and ser73[4]. Scaffold proteins can provide an assembly site for such specialized protein interactions. These scaffold proteins usually do not contain any intrinsic enzymatic activity but possess a structure that enables them to recruit different factors of a specific pathway simultaneously[5]. But, although scaffold proteins increase specifity of individual signaling cascades, they act at the expense of signal amplification. Spatial localization of signaling molecules is another device to augment specificity in signal tranduction. E.g., MEKK1 colocalizes with elements of the cytoskeleton and cytoskeletal rearrangements may stimulate MEKK1 activity[4]. Finally, the duration of the signal can strongly influence the direction of the various pathways[6]. However, once activated, the MAPK cascade enables the cell to respond to environmental changes in a prompt and ordered fashion[3].

The Ras/ Raf/ MEK1/ ERK1/2-pathway.
The classic ERK1/2 cascade may serve as a prototype of the other MAPK cascades. It is discussed extensively here to improve the understanding of the basic principle of MAPK signal transduction pathways. The Ras/ Raf/ MEK1/ ERK1/2 cascade is activated in response to many mitogenic stimuli, such as EGF, PDGF, thromboxane A2, angiotensin II, TGF, insulin, LPS, osmotic stress and adherence of monocytes and endothelial cells.
Ras is a small guanosine tri phosphatase- (GTP-ase) that is activated through its interaction with the Grb2- Sos (son of sevenless) complex, where Sos catalyzes the dislocation of GDP with the subsequent formation of Ras- guanosine tri phosphate (GTP) complex. Different Ras isoforms and mutations have been observed, which possess varying abilities to activate downstream signaling pathways. In its GTP-bound state, Ras recruites Raf to the membrane. The known members of the mammalian Raf gene family are Raf-1, A-Raf and B-Raf. Raf is one of the MAPKKK kinases and subsequently activates its downstream MAPKK, MEK1. Raf, however, is not the only inducer of MEK1 and ERK1/2 activation. All MAPKKK family members, with the exception of MEKK4, have the potential to activate MEK1. By contrast, Raf is unable to activate other MAPKKs[5]. The ERKs are characterized in two isoforms, ERK1 and ERK2 (ERK1/2), which are sometimes referred to as p44/42 MAP kinases.

Because ERKs, like other MAPKs, are activated by phosphorylation, protein phosphatases dephosphorylating MAPKs are key elements in controlling ERK1/2 activity[1]. The duration as well as the magnitude of the ERK1/2 signal is a critical factor in determining the response of a certain type of cell to changes of the extracellular environment, i.e. a transient ERK1/2 signal activates other transcription factors than a sustained ERK1/2 activity[7]. In keratinocytes, a mitogenic stimulus such as provided by growth factors results in a relatively strong, transient activation of Raf/ ERK1/2. Then, after minutes, the signaling is downregulated. Such a transient signaling may dictate modulation in the level of $P21^{cip1/waf1}$ expression, resulting in cell cycle progression[7]. It has been reported that a sustained ERK1/2 signaling may occur independently of Ras, through a newly discovered GTP-ase, Rap1. Convergence towards ERK1/2 activation occurs on the level of Raf. Sustained activation may also occur through the cooperative activation of different receptors e.g., the EGF- and the integrin receptor. It has been demonstrated in keratinocytes that such a sustained Raf/ ERK1/2 activation, induces a persistently high level of $p21^{cip1/waf1}$ and a subsequent G1 arrest[7]. In the nucleus, ERKs can phosphorylate and activate a number of transcription factors such as c- fos, Elk-1, NFκB and Jun. Fos and Jun family members homo- or heterodimerize to an AP1 transcription promotor complex. Dependent on the composition of the complex, differential gene transcription and protein production occurs.
Once ERK1/2 have been activated they can also target cytoplasmic - or cytoskeletal proteins. Cross talking between other MAPKs such as p38, but also with other signaling pathways like the TGFβ/ SMAD and the PI3K/ Akt have been reported[8-10]. By its broad spectrum activation program, the Ras/ c-Raf1/ MEK1/ ERK1/2/ MAPK signal transduction pathway is involved in most cellular processes like proliferation, cell cycle arrest and apoptosis.

The Ras/ MEKK1/ MEK4/ JNK/ SAPK pathway
The c-Jun N-terminal kinase (JNK) was originally identified as the UV-induced factor responsible for phosphorylation and thus activation of the transcription factor c-Jun[11]. JNKs are also characterized as stress-activated protein kinases (SAPK) on the basis of their activation in response to inhibition of protein synthesis[12]. Three genes JNK1, JNK2 and JNK3 with 12 possible isoforms derived from alternative splicing products have been described[13]. Environmental stress, radiation, growth factors and endotoxins induce activation of JNK/SAPKs. Regulation of the JNK/ SAPK pathway is extremely complex and is influenced by many MAPKs. There are e.g., 13 MAPKKs that can regulate the JNK/SAPKs. This diversity allows a wide range of stimuli to activate this MAPK pathway[14]. JNK/SAPKs are considered to be important in controling programmed cell death or apoptosis.

The Ras/ MEKK1/ MEKK6/ p38 pathway
P38 is recognized as the MAPK that is activated in response to physiologic stress, osmotic stress, LPS and UV exposure[3]. The p38 MAPK family has been shown to consist of four different isoforms, p38α, -β, -γ and –δ[15]. Different activation and substrate specifity of each p38 isoform results in their different physiological

functions. It has been shown that p38α and p38β are ubiquitously expressed, while p38γ and p38δ are expressed in a more tissue specific manner. p38γ expression, e.g., has not been detected in the epidermis, while p38δ plays a key role in epidermal differentiation[8].
Besides activation via the MAPK signaling cascade, the p38α, but also the JNK kinase have been shown to be activated by the protein complex TAK1/2/ TAB1, which is part of the interleukin-1 cytokine signaling pathway[6,16]. Because TAB1 has no known catalytic activity, it appears to be an adaptor or scaffolding protein[14]. This is an important observation, which indicates that inflammatory cytokines, or other adaptor proteins, may be involved in the activation of different MAPK pathways through one signaling module. Complex formation e.g., between two MAPKs, p38 and ERK, has also been reported, underlining the complexity of the system[8]. A number of downstream targets of p38 have been demonstrated. Cytoplasmic substrates of p38 include different protein kinases which act on the translational as well as the transcriptional level. In the nucleus, p38 regulates the activity of a number of transcripion factors such as ELK1, p53, NFκB and AP-1[5,8,17]. However, p38 is generally considered to be the MAPKinase, which is dominant in the regulation of keratinocyte terminal differentiation via the AP-1 human involucrin promoter[8,18].

pAkt signaling

Recent studies have revealed a burgeoning list of Akt/ PKB substrates implicated in oncogenesis, nutrient metabolism, transcriptional regulation and cell survival[19]. Among its pleiotrophic effects, activated Akt/ PKB is a well established survival factor and in this chapter we will discuss the molecular mechanism of its function in regulating cell survival particularly. In mammals, three Akt/ PKB genes have been identified, termed Akt1/ PKBα, Akt2/ PKBβ and Akt3/ PKBγ. Akt/ PKB are the downstream effector kinases of phosphoinositide 3 kinase (PI3K), which is activated by growth factors via a tyrosine kinase receptor and via the G-protein-coupled receptors (Fig.2). Following ligand binding, PI3K is recruited to the cell membrane and activated. Then PI3K interacts with and phosphorylates phosphatidylinositoldiphosphate (PIP2), which results in generation of phosphatidylinositoltriphosphate (PIP3). PIP3 does not activate Akt/ PKB directly but instead appears to recruit Akt/PKB to the plasma membrane and to alter its conformation. This allows subsequent phosphorylation by phosphoinositide-dependent kinase-1 (PDK1). The Akt/ PKB protein has two phosphorylation sites, Thr308/309 and Ser473/474, of which Thr308/309 phosphorylation is necessary for Akt/ PKB activation while Ser473/474 is required for maximal activity[20]. Activated Akt/ PKB is then released from the membrane and translocates to both the cytosol and the nucleus[21].
Different reports have suggested that pAkt/ PKB can be activated in a PI3K-independent way. It has been shown that cyclic adenosin mono phosphate (cAMP) inducing agents such as prostaglandin-E1 were able to activate Akt/ PKB through Protein Kinase A (PKA). Although the mechanism is not quite clear, it appears that dual phosphorylation of Akt/ PKB is not required for PKA mediated pAkt/ PKB activation. It has also been shown in vitro that Akt/ PKB can be activated by Ca2+/

calmodulin-dependent kinases[19]. Moreover, it has been indicated that pAkt/ PKB can be activated by cellular stress and heat shock proteins[19]. Once activated, the control of cellular survival of Akt/ PKB occurs via direct and indirect effects on the apoptotic pathway. These effects are: post transcriptional regulation, activation of transcription factors and metabolic interaction.

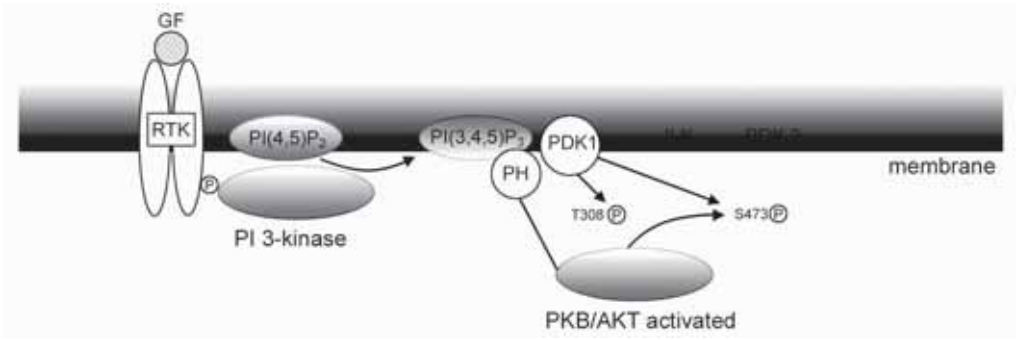

Figure 2.
Akt signaling pathway, activation of different cell-surface receptors, such as tyrosin kinase receptors, induce production of second messengers like PIP3, phosphatidylinositol 3,4,5-trisphosphate, that convey signals to the cytoplasm from the cell surface. PIP3 signals activates the kinase PDK1, 3-phosphoinositide-dependent protein kinase-1, which in turn activates the kinase Akt, also known as protein kinase B. The Akt/ PKB protein has two phosphorylation sites, Thr308/309 and Ser473/474.

There are numerous post-transcriptional Akt/ PKB-mediated cellular survival mechanisms of which the most important are discussed below. One important mechanism includes phosphorylation and de-activation of different pro-apoptotic proteins such as the BAD protein[22], caspase-9[23], and apoptosis- mediating MAPKinases. Akt/ PKB also initiates indirect de-activation of pro-apoptotic proteins such as apoptosis signal-regulating kinase (ASK1)[24]. Akt/ PKB-induced phosphorylation may also comprise activation of anti-apoptotic proteins of which murine double minute 2 (MDM2) is the most essential one[25]. Other mechanisms include activation of anti-apoptotic proteins like NFκB, by binding to and phosphorylation of their inhibitors.
BAD is a pro-apoptotic member of the BCl-2 family, phosphorylation of BAD may occur at two critical sites, Ser112 and Ser136. Phosphorylation at each site is sufficient for association of BAD to 14-3-3. However, full inactivation of the protein is only induced when both serines are phosphorylated[22]. This synergistic phosphorylation is driven by the MAPK signaling pathway at Ser112- and by Akt/ PKB-mediated phosphorylation on the Ser136-site[22].
Caspase-9 is called a death protease and acts as one of the direct effectors of apoptosis. Akt/ PKB-induced phosphorylation of caspase-9 has been shown to diminish the activation of its target execution caspases[23].
ASK1 is one of the MAPKKKs that interacts with and is phosphorylated by Akt/ PKB on Ser83. This results in a decreased ASK1 mediated signaling to JNK/ SAPK and a suppressed susceptibility to apoptosis[24].

Indirect inactivation of ASK may also prevent apoptosis. The cell growth inhibitory activity of $p21^{Cip1/WAF1}$ is strongly correlated with its nuclear localization. However, Zhou et al. have shown that Akt/PKB phosphorylation of Thr145 in $p21^{Cip1/WAF1}$ triggers its cytoplasmic localization[25]. Cytoplasmic $p21^{Cip1/WAF1}$ then forms a complex with ASK1 which, indirectly, results in resistance to apoptosis[26].
MDM2 is an oncoprotein localized in the cytoplasm in a complex with Akt/ PKB. After growth factor stimulation, Akt/ PKB phosphorylates MDM2 on two residues Ser166 and Ser 186. Then MDM2 dissociates from the complex and enters the nucleus where it binds to p53. The MDM2-p53 complex subsequently shuttles to the cytoplasm where p53 is targeted for ubiquitine proteasome-mediated degradation[27]. Under certain circumstances like cellular stress or UV-radiation, p53 has been reported to mediate cell death. Therefore, Akt/ PKB could support cellular survival by promoting degradation of p53.
It has been shown that Akt can activate the transcription factor NF-κB and that this blocks apoptosis induced by certain stimuli. The mechanism whereby Akt activates NF-κB has been controversial, with evidence supporting induction of nuclear translocation of NF-κB via activation of IκB kinase activity and/or the stimulation of the transcription function of NF-κB[28-31]. It has also been demonstrated that Akt targets the transactivation function of NF-κB in a manner that is dependent on IκB kinase activity and on the MAPK p38[32]. These disparate observations point to deficiencies in the understanding of the Akt/ PKB-mediated NF-κB activation. However, it is generally accepted that Akt/ PKB is involved in NF-κB transcription of pro-survival proteins, such as Bcl-xL and caspase inhibitors[19].
Recent studies have shown that Akt/ PKB can regulate cellular survival through transcriptional factors that are responsible for pro- as well as anti-apoptotic genes. The most known are Forkhead (FH). Akt/ PKB can directly phosphorylate all four isoforms of FH. The phosphorylated FH proteins can promote cell survival by inhibiting the activity of a number of FH target genes. These target genes are usually extracellular ligands and important in promoting apoptosis. The most common are the FAS-ligand, TNF-related apoptosis-inducing ligand (TRAIL) and TNF receptor type 1 associated death domain (TRADD)[33].
A major physiologic function of Akt/ PKB is the regulation of cell metabolism. When high levels of insulin are present, Akt/ PKB phosphorylates glycogen synthase 3 (GSK-3), which inhibits its function. This promotes the storage and utilization of glucose. It has been hypothesized that the inhibition of GSK3 is protective against growth factor-deprived apoptosis[21].

TGFβ signaling

Transforming growth factor β (TGFβ) is the prototype of a large family of cytokines that regulate a wide variety of cellular processes including cell proliferation, cell differentiation, cell motility and extracellular matrix production. The TGFβ family includes a large number of related proteins including bone morphogenetic proteins (BMP). The effects of TGFβ on cell growth control are complex and can vary dramatically depending on the target cell type and the presence of other growth factors[34]. TGFβ-related factors signal through ligand binding to the type II TGFβ

receptor and, after forming a heterodimer with the type I TGFβ receptor, signal propagation occurs by phosphorylation of the receptor-specific (R) Smads (Fig.3).

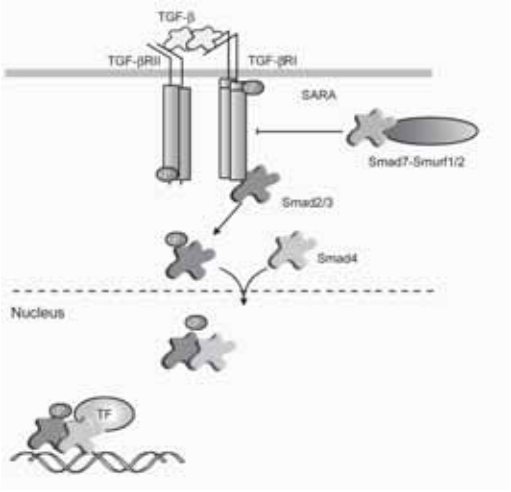

Figure 3.
TGFβ-related factors signal through ligand binding to the type II TGFβ receptor and, after forming a heterodimer with the type I TGFβ receptor, signal propagation occurs by phosphorylation of the receptor-specific (R) Smads. The TGFβ part of the family mediates signaling by activation of the R-Smads, Smad2 and Smad3. The phosphorylated R-Smads then oligomerise Smad4 and translocate to the nucleus, where they regulate the transcription of target genes. The inhibitor Smads (I-Smads), Smad6 and Smad7, function as a broad spectrum intracellular antagonists of TGFβ family signaling. I-Smads bind to the activated receptor complex, in competition with the R-Smads. In addition, Smad7 mediates docking of the ubiquitine ligases Smurf1 and Smurf2 to the TGFβ receptor. This causes ubiquitination and proteasomal degradation of the receptor.

The TGFβ part of the family mediates signaling by activation of the R-Smads, Smad2 and Smad3. BMP signals through activation of the other R-Smads: Smad1, Smad5 and Smad8[35]. The phosphorylated R-Smads then oligomerise with the common-mediator Smad (co-Smad, Smad4), translocate to the nucleus and regulate the transcription of target genes[36]. The inhibitor Smads (I-Smads), Smad6 and Smad7 function as broad spectrum intracellular antagonists of TGFβ family signaling[37]. Expression of inhibitory Smads is induced by multiple stimuli, including EGF and various members of the TGFβ family. I-Smads bind to the activated receptor complex, in competition with the R-Smads. In addition, Smad7 mediates docking of the ubiquitine ligases Smurf1 and Smurf2 to the TGFβ and BMP receptors. This causes ubiquitination and proteasomal degradation of the receptors. Smad7 counters most of the TGFβ-regulated processes in the cell like growth inhibition via $p21^{Cip1/Waf1}$ and production of extracellular matrix proteins. Contradictory, Smad7 expression is necessary for TGFβ-induced apoptosis[38]. The association of the Smad complexes with transcription factors and transcriptional co-activators/co-repressors in the nucleus regulates transcriptional control by TGFβ. TGFβ modulates several other signaling pathways such as the JNK MAPK, which can either be activated or inhibited by TGFβ[39]. Differential activation of ERK1/2 and p38 by TGFβ has also been reported [39,40]. Moreover, in keratinocytes, epidermal mesenchymal transition (EMT) is induced by TGFβ 1 through the activation of both ERK1/2 and p38 MAPKs[41]. Interestingly, co-treatment of cells with EGF enhanced the activation of these MAPKs[41]. In this report, phosphorylation of JNK could not be detected. However, specific inhibitors of MEK1, p38 and JNK all blocked EMT, indicating that activation of all three pathways

was required for TGFβ 1-induced EMT[41]. Crosstalking to the PI3K-Akt pathway has also been reported. It has been demonstrated that TGFß phosphorylates Akt in a PI3K-dependent manner, leading to TGFβ-mediated EMT and cell motility[42].

p53 in repair

Activation of p53

The p53 tumour suppressor protein plays a pivotal role in essential cellular processes like apoptosis, cell cycle control, senescence, differentiation and neoplastic transformation. The function of p53 involves mainly the prevention of the accumulation of genetic alterations by initiating signaling pathways to either DNA repair by growth arrest/ senescence or by eliminating cells by apoptosis. The p53 gene can be activated in many stress responses like DNA damage, illegitimate activation of oncogenes, hypoxia and inflammatory cytokines triggering[43]. In the absence of cellular stress p53 expression is maintained at a low level. At present, multiple lines of evidence indicate that one of the key mechanisms by which p53 functions is regulated through control of the MDM2 protein. MDM2 has been shown to inhibit p53 activity in at least two distinct molecular mechanisms. This may occur by binding to the N-terminal transactivation domain of p53, which blocks interactions with other proteins necessary for p53-dependent regulation of gene expression[43]. Another mechanism, essential under non-stress conditions, is the targeting of p53 for ubiquitination which leads to proteasomal degradation[45,46]. Since p53 can also associate to p53 binding sites within the MDM2 promoter, it can trigger MDM2 expression. This is an important negative autoregulatory feedback mechanism in p53-MDM2 interaction[43,44]. In some cancer cells, mutant p53 does not induce MDM2 gene expression. In that situation, mutant p53 is not degraded and its half-life in cells is prolonged[47]. The regulation of p53 stability is a complex process that is dependent of many different forms of stress. Many pathways can be used to allow stabilization of p53, such as phosphorylation, inhibition of MDM2 synthesis or cytoplasmic sequestration of p53[45,48]. Another important regulatory mechanism of p53 stabilization via MDM2 inhibition is the binding of the alternative reading frame (ARF). This binding inhibits the p53- targeting for ubiquitination[49-51]. The consequence of ARF expression is the efficient stabilization and activation of p53. ARF plays an important role in the induction of p53 in response to oncogenic activation, eliminating cells with proliferative abnormalities[43]. In addition to the inhibition of MDM2 by ARF, it has recently been established that the AKT/PKB kinase can also be engaged in MDM2 inhibition (see Akt section). There are also MDM2-independent mechanisms for p53 degradation, of which JNK, by targeting p53 for ubiquitination, appears to be the most important[52] .

Activation by p53

The molecular basis for the differential activation of particular sets of target genes by p53 is not fully understood. Multiple molecular mechanisms most certainly contribute to p53 target gene selectivity. Studied intensively is the covalent modification of the p53 protein by phosphorylation. The most important kinases

involved in p53 phosphorylation include casein kinase, CHK1 and 2, DNA-dependent protein kinase (DNA-PK) and JNK[53-55]. These kinases also phosphorylate MDM2 in vitro, suggesting a regulatory role for these modifications[56-58]. It has been suggested that the phosphorylated p53 protein undergoes some conformational changes, which alters its DNA-binding specifity. In line with this, it has been demonstrated that phosphorylation on specific residues of p53 alters its DNA binding preference in vitro[43]. But, the in vivo relevance of this finding has been questioned[43]. Besides modifications of the p53 protein, the transcription of particular target genes appears to be determined by interaction of p53 with other proteins. These proteins may be transcriptional coactivators like p300, CBP, PCAF and E2F1 which have been shown to enhance p53-mediated transcription[59]. The need for additional p53 partners may be of particular importance for genes with a low-affinity p53 binding site (p53BS). It is of interest that many, if not all, pro-apoptotic p53 target genes harbour p53BS of rather low binding affinity. Thus, this subclass of genes may rely more heavily on cooperation of p53 and its co-activators, whereas cell cycle inhibitory genes may be turned on by p53 as a default option[43]. The question how p53 chooses between induction of apoptosis versus induction of a viable growth arrest has received great attention. As it appears now, p53 is not the only determinant that influences this choice. The phenotype of the cell, the extra cellular stimuli and the type of stress and its intensity are of great importance for the direction of p53 transcriptional activities[60]. With respect to the phenotype, different cell types may respond to the same apoptotic stimulus with either apoptosis, or cell cycle arrest[61]. This might be due to their differential ability to induce pro-apoptotic proteins of the Bcl-2 family, like Bax, Noxa and Puma[43]. It might also be possible that the difference in apoptotic threshold is a reflection of the biology of the cells involved. Cells with a high turnover, like T cells, must respond quickly to death stimuli in order to limit the immunological response[60]. In general, activation of p53 in normal cells results in cell- cycle arrest or senescence, whereas in malignantly transformed cells p53 usually promotes apoptosis[61]. Moreover, extracellular stimuli, such as cytokines and growth factors can protect cells from apoptotic response to cell death stimuli or DNA damage by p53-promoted growth arrest[62].

The ability of p53 to promote apoptosis has been studied extensively and multiple pathways have been identified. However, to what extent each pathway contributes to the apoptotic activity of p53 remains a controversial matter. One of the most prominent pathways is the mitochondrial pathway which is involved in the transcription of the pro-apoptotic proteins Bax, Noxa and Puma and their transport to the mitochondria[43,63]. This action promotes loss of the mitochondrial membrane potential and cytochrome c release, which results in the formation of the apoptosome, a holoenzyme consisting of cytochrome c, APAF1 and pro-caspase 9[63]. Pro-caspase 9 is one of the members of a family of programmed cell death executioner cysteine proteases, which are called caspases[63]. The apoptosome promotes cleavage of pro-caspase 3 to its active form, activated caspase 3. Activated caspase 3 is a so-called effector caspase, which cleaves the inactive part from

caspase-activated DNAse (CAD). This is the final step in DNA degradation[63]. Among the caspases, activated caspase 3 is considered to be an important marker of ongoing apoptosis[64]. Another important pro-apoptotic activity of p53 implies the membrane death receptor induced pathway of apoptosis. Expression of at least two of the death receptors FAS/APO1 and DR5/KILLER and one of the death receptor ligands FASL, have been observed to be upregulated by p53[65,66]. Activation of death receptors by their ligands (FAS by FASL and TRIAL by DR5) results in trimerization and recruitment of intracellular adaptor molecules which initiate the caspase cleavage cascade and apoptosis. Moreover, p53 can also promote apoptosis by the negative regulation of the integrin-associated survival signaling[67,68]. A differential role in p53-mediated apoptosis is played by the transcription factor NF-κB. Its positive effects have been demonstrated[69], but it is also known as a mediator for expressing survival genes[70].
The function of p53 in the control of cell cycle arrest appears to be primarily mediated by genes, dominated by $p21^{waf1/cip1}$, and will be discussed below. p53 can also trigger growth arrest in a p21-independent way, by the preventing the activation of the cyclin-dependent kinase (CDK)2/cyclin A kinase, which is required for G1/S transition[71]. Another p21-independent, p53 mediated growth arrest is the induction of 14-3-3s, and to some extent that of the GADD45 gene[43,72], which both lead to a G2 arrest[43].

$p21^{cip1/waf1}$ in cell cycle control

The $p21^{cip1/waf1}$ protein has been shown to play an essential function in mediating G1 arrest in response to DNA damage as well as in blocking the re-entry of G2 cells into S phase. $p21^{cip1/waf1}$ transcription is usually p53-dependent, but p53-independent upregulation of $p21^{cip1/waf1}$ has also been reported[73]. $p21^{cip1/waf1}$ levels are also regulated post-transcriptionally. $p21^{cip1/waf1}$ is subject to proteosome-dependent degradation. This degradation can be prevented by interactions with proteins, that can bind to the c-terminal domain of $p21^{cip1/waf1}$, i.e., the binding site of the proteasome[74]. There are different proteins with this binding capability such as PCNA, cyclins or MAPKs[73,74]. Increased $p21^{cip1/waf1}$ expression is not necessarily linked to growth arrest, as it also occurs when cells are growth-stimulated, e.g., after growth factor exposure[75]. These apparently conflicting findings can be reconciled by the fact that $p21^{cip1/waf1}$ plays a dual function as both an inhibitor of cyclin/ CDK activity, and a positive modulator of cyclin/ CDK complex formation and nuclear localization. During physiological mitogenic stimulation, expression of D-type cyclins is induced and gives rise to the formation of cyclin D/ CDK4 and cyclin D/ CDK6 complexes (Fig. 4). These complexes are phosphorylated by cyclin activating kinase (CAK) and become components from a tetrameric complex which consists of the cyclin D, CDK4/6, $p21^{cip1/waf1}$ and PCNA. The active cyclin D complex phosphorylates the retinoblastoma protein (Rb). Rb is subsequently double phosphorylated by the cyclin E/ CDK2/ $p21^{cip1/waf1}$ / PCNA complex, which on its turn has been phosphorylated by CAK. Once Rb has become hyperphosphorylated, the transcription factor E2F1 is released from inhibition by Rb and the expression of genes required for the S phase is induced[73].

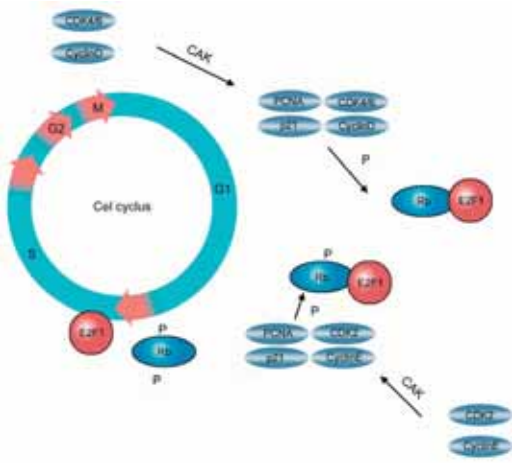

Figure 4.
The cell cycle and the involvement of different protein complexes in its progression. When expression of D-type cyclins is induced, the formation of cyclin D/ CDK4 and cyclin D/ CDK6 complexes is initiated. After phosphorylation of these complexes by cyclin activating kinase (CAK) they form a tetrameric complex with $p21^{cip1/waf1}$ and PCNA. This complex phosphorylates the retinoblastoma protein (Rb). Rb is subsequently double phosphorylated by the cyclin E/ CDK2/ $p21^{cip1/waf1}$/ PCNA complex. This complex has also been formed after phosphorylation by CAK. Once Rb has become hyperphosphorylated, the transcription factor E2F1 is released from inhibition by Rb and the expression of genes required for the S phase is induced.

Thus, at low levels, $p21^{cip1/waf1}$ functions as an assembly factor for cyclin D/ CDK4/ 6/ PCNA complexes.
The induction of D-type cyclins in early G1 recruits $p21^{cip1/waf1}$ into these active complexes. By insertion into this complex, free $p21^{cip1/waf1}$ is prevented from its inhibitory effects on cyclin E/ CDK2/ PCNA, which promotes progression through G1[73]. Consistent with its requirement for progression through G1, $p21^{cip1/waf1}$ expression level is very low in quiescent cells. High and sustained levels of $p21^{cip1/waf1}$, however, inhibit the activity of all CDKs, especially the cyclin E/ CDK2 complexes. In contrast to cyclin E/ CDK2, $p21^{cip1/waf1}$ is unable to inhibit cyclin E/ CDK4 at equimolar amounts and may require higher stoichiometric amounts to achieve inhibition[73]. The growth arresting activity of $p21^{cip1/waf1}$ is consistent with its induction during cell differentiation, in growth arrest by TGFβ and in senescent cells.
The amino-terminal domain of $p21^{cip1/waf1}$ is both necessary and sufficient to inhibit cyclin/ CDK activity in vitro and in vivo. The unique carboxy-terminal domain of $p21^{Cip1/waf1}$ associates with the proliferating nuclear antigen (PCNA), a subunit of δ DNA polymerase. Besides involvement in the quaternary complex of cyclin/ CDK/ $p21^{cip1/waf1}$ and PCNA, $p21^{cip1/waf1}$ has also been shown to form a binary complex with PCNA. This binary $p21^{cip1/waf1}$-PCNA complex is capable to regulate DNA replication directly, without affecting DNA repair. The association of the carboxy-terminal domain of $p21^{cip1/waf1}$ with the proliferating cell nuclear antigen (PCNA) competes with the interaction of the PCNA-δ DNA polymerase complex with DNA- metabolizing enzymes, like (DNA cytosine-5) methyltransferase (MCMT) and FEN1(Flap endonuclease 1)[74]. $p21^{cip1/waf1}$ is involved in different anti-apoptotic processes. Inhibition by $p21^{cip1/waf1}$

of CDKs have been reported to be a necessary step to prevent apoptosis, either upstream or downstream of caspase activation[74] The possibility that the anti-apoptotic effects of $p21^{cip1/waf1}$ are due to cyclin/ CDK inhibition is supported by the fact that similar effects can be observed with expression of CDK dominant negative mutants. However, a second mechanism by which $p21^{cip1/waf1}$ could protect cells from apoptosis is through its effects on molecules more specifically involved in the apoptotic process, such as caspases 8 and -10, caspase 3, SAPKs or MEKKs (ASK1)[74]. Thus, the induction of $p21^{cip1/waf1}$ in response to apoptotic stimuli arrests cell cycle progression via the inhibition of CDKs, but may also help to prevent apoptosis.
The $p21^{cip1/waf1}$ protein has also been shown to have a role in irreversible cell cycle arrest: senescence, which may result from critically shortened telomeres[76]. Some of these functions are at least partially exerted through activation of the p53 transcription factor. However, senescence and p21 transcriptional induction also occur in p53-defective HaCaT (immortalized keratinocyte) cells[77].
Besides the functions of $p21^{cip1/waf1}$ in cell cycle control, apoptosis and senescence, this molecule plays an unexpectedly complex role in differentiation. Different studies in which cellular differentiation has been induced e.g., by increasing cellular calcium levels, or by interruption of cell-extracellular matrix contacts or by 12-o-tetradecanoylophorbol-13-acetate (TPA application) have shown a concomitant increase in $p21^{cip1/waf1}$ expression[78,79]. However, $p21^{cip1/waf1}$ null mice develop normally[80], which may indicate that other proteins such as p27, may complement $p21^{cip1/waf1}$ function.
Recently, it has been reported that $p21^{cip1/waf1}$ plays a key role in restricting the number of keratinocyte stem cell populations, as well as further downstream, determining the irreversibility of stem cell differentiation[81]. The interconnection of $p21^{cip1/waf1}$ and the Notch family of cell surface receptors, inserts the $p21^{cip1/waf1}$ protein in the stem cell/ transit amplifying cell regulatory mechanism[82].
Additionally, endogenous Notch1 activity is required for induction of $p21^{cip1/waf1}$ expression in differentiation. In fact, in mouse keratinocytes, the p21 gene is a direct transcriptional target of Notch1 activation.
Altogether, $p21^{cip1/waf1}$ can considered to be a mediator in the transition between growth and growth arrest. This may imply that $p21^{cip1/waf1}$ is likely to play an essential role in restricting the number of keratinocytes with high growth potential.

A central question of differential signaling in a given system, is whether multiple substrates can be activated simultaneously. Thus, several downstream signaling pathways may function in parallel, perhaps acting as a multipathway signaling unit. The proteins and protein signaling pathways described in this thesis may also be subjected to such complex interactions.

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Chapter 3

3

Cholesteatoma epithelium is characterized by increased expression of Ki-67, p53 and p21,with minimal apoptosis

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Abstract

Objective- to investigate differences in cell proliferation, cell cycle arrest and apoptosis between cholesteatoma and control skin.

Materials and Methods- Immunohistochemical sections of 15 cholesteatoma and 15 paired control retro-auricular skin samples were examined for Ki-67, p53, p21 and active caspase 3 using image analysis, as well as for DNA fragmentation.

Results- The retro-auricular skin samples contained 5.7± 3.6% of Ki-67-positive cells and showed a normal expression pattern. In the cholesteatoma epithelium 11.7± 9.5% of the cells were Ki-67-positive and these cells were dominantly expressed in the basal and parabasal cell layers. Retro-auricular skin contained 5.8± 5.4% p53-positive cells and 1.0± 0.9% p21-positive cells. In the cholesteatoma epithelia 17.8± 12.3% of the cells were p53-positive and 14.3± 11.6% were p21-positive. The expression of Ki-67, p53 and p21 differed significantly between the two groups ($p<0.05$). In the cholesteatoma epithelium a positive correlation was found between p53 and p21 expression ($p= 0.016$). Active caspase 3 positivity and DNA fragmentation were rarely seen in the cholesteatoma epithelium.

Conclusion- Our results indicate that increased cell proliferation in cholesteatoma epithelium is accompanied by an increase in p53 and p21 protein levels whilst apoptosis is minimal.

Keywords: *active caspase 3, apoptosis, cholesteatoma, immunohistochemistry, Ki-67, p21, p53, terminal deoxynucleotide transferase-mediated dUTP nick-end labeling.*

Introduction

Cholesteatoma is a benign, but destructive middle ear tumor, characterized by a hyperproliferative epithelium with progressive accumulation of keratin. Homeostatic growth of keratinocytes depends on the controlled coordination of cell proliferation and programmed cell death. However, in cholesteatoma this process appears to be unbalanced. It has been shown that proliferating cells in the cholesteatoma epithelium are dislocated[1] and that the level of expression of proliferation markers is aberrant[2]. Cell viability or cell death is determined by the activity of a complex intertwined gene family of cell death stimulators and inhibitors. One of the most important members of this family is the p53 protein. It can activate a cascade of programmed cell death executioner cysteine proteases, known as caspases[3,4]. Among these, activated caspase 3 is considered to be an important marker of ongoing apoptosis[5]. Besides initiating apoptosis, p53 can also perform other functions such as controlling the initiation of mitosis[6]. One part of the mechanism by which p53 blocks cells at the G1 checkpoint involves upregulation of p21, a cyclin-dependent kinase inhibitor[7]. This dual function, the regulation of either apoptosis or cell cycle arrest, makes the role of p53 in the formation and development of cholesteatoma of particular interest. In the literature, however, there is controversy regarding the level of expression of p53[2,8,9] and the degree of apoptosis in the cholesteatoma.[9-11]. There is also a difference of opinion concerning

the degree of cell proliferation in cholesteatoma with respect to the expression of p53[2,8,9]. Furthermore, in cholesteatoma, studies of cell cycle arrest by means of p21 expression have not previously been published. The present study was instituted in order to compare the degree of cell proliferation, cell cycle arrest and apoptosis in the cholesteatoma epithelium. For this purpose we have determined the expression of: p53, the proliferation marker Ki-67, p21 and activated caspase 3. As a second measure of apoptosis we determined DNA fragmentation by means of the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) technique and calculated the percentage of apoptotic cells in the tissue samples. The results obtained in cholesteatoma tissue were compared to those in paired control samples from retro-auricular skin.

Materials and methods

Clinical and histopathological data

Cholesteatoma specimens from the pars flaccida and biopsies of retro-auricular skin were obtained from fifteen patients, and immediately placed in phosphate buffered saline. The Committee of Medical Ethics of the Leiden University Medical Center approved the protocol. The specimens were prepared for histological examination by fixation in 4% buffered formaldehyde for 20 h. and dehydration in ethanol and were then embedded in paraffin wax.

Immunohistochemistry

Serial sections (4μm) were taken from each tissue block. The first and the last sections were stained with hematoxylin- eosin (HE). Subsequent sections were immunostained with either p53, p21, Ki-67 or active caspase 3 antibodies. The penultimate section was used for in situ detection of fragmented DNA using the TUNEL technique. Sections from the same tissue served as negative controls, i.e. the primary antibody was omitted. The expressions of p53, p21, Ki-67 and active caspase 3 were determined using an indirect immunoperoxidase method. Anti-p53 (DO7), anti-p21(WAF1) and anti- Ki-67(MIB-1) were monoclonal antibodies purchased from NeoMarkers Inc (Fremont, CA), Oncogene Research Products (Cambridge, UK) and Immunotech (Marseilles, France), respectively. The dilutions used were 1: 1000, 1:200 and 1: 400, respectively. The active caspase 3 (3p20) polyclonal antibody was purchased from Promega (Madison, WI) and diluted 1: 400. Sections from coloncarcinoma (p53), sigmoid colon (p21), tonsil (Ki-67), thymus and castrated rat prostate (active caspase 3) were used as positive controls. To inactivate endogenous peroxidase, the deparaffinized sections were treated with methanol containing 3% H_2O_2 for 20 minutes. After rehydration, the sections were subjected to microwave antigen retrieval in citrate buffer (0.01M, pH 6.0) for 12 min[12]. The sections assigned for p21 and active caspase 3 antibody treatment were also subjected to pepsin antigen retrieval (4% pepsin in 3mM HCl) for 5 min at room temperature (RT).[13] All sections were incubated with the primary antibody overnight at RT and then washed in PBS. The specimens were incubated with appropriate biotinylated secondary antibodies for 30 min at RT, washed and

subsequently incubated with peroxidase-conjugated streptavidin at RT for 30 min. They were then treated with 3,3′ di- aminobenzidine (DAB) chromogen containing 0.02% H_2O_2 and counterstained with HE for 1 min.

TUNEL staining

Fragmented DNA was monitored by means of an adapted fluorochrome/ enzyme immunoassay, a modification of the method of Negoescu et al[14]. We used samples of thymus and castrated rat prostate as positive controls. After deparaffination and rehydration, the sections were pretreated with microwave irradiation (750W, 0.1M citrate buffer) for 1min and rapidly cooled. After washing with Tris-buffered saline (TBS), they were incubated with blocking buffer (0.1 M Tris-HCl, 3% bovine serum albumin and 20% newborn calf serum) for 15 min at RT. The slides were rinsed with TBS and the specimens were incubated with labeling mixture in a humid atmosphere at 37ºC for 60 min. The labeling mixture contained terminal deoxynucleotidyl transferase and fluorescein-d uridine triphosphate [In Situ Cell Death (ISCD) detection kit; Roche Diagnostics, Mannheim, Germany]. Transferring the slides to stop buffer (300mM sodiumchloride, 30 mM sodiumcitrate) for 15 min at RT terminated the reaction. After washing with TBS, incubation with blocking buffer was repeated and the slides were then rinsed again in TBS. Fluorescein was labeled with peroxidase-conjugated rabbit anti-fluorescein isothiocyanate (DAKO, Glostrup, Denmark), diluted 1:50 with blocking buffer for 30 min at RT. This modification was used because pilot studies with the ISCD kit converter revealed false positives. The sections were stained with DAB chromogen for 1 min and counterstained with HE.

Morphometric analysis of immunohistochemical data

For each of the immunohistochemical markers studied, DAB positive staining was quantified using an image analysis system (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). The microscope was a Leica DMLB with a Leica DC 200 digital camera. The computer-assisted system used to determine the immunohistochemical positive staining has been described elsewhere[15]. 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 stains, were used. The epithelial compartment was delineated on the screen and positive and negative cells were counted automatically. In each section >1000 cells were counted and the percentage of positive cells was determined.

Data analysis

Data are expressed as means ± SD. In order to compare the means of paired variables, the paired samples t-test was used, with a level of significance of $p < 0.05$ was performed. The Pearson two-tailed correlation test was used to calculate possible correlations. Correlation was considered significant at the 0.05 level. The SPSS 10 software package (SPSS, Chicago, IL) was used for the calculations.

Results

Histopathological findings

In 10/15 cholesteatoma samples we found inflammatory cells and newly formed blood vessels in the connective tissue. In one tissue sample there was insufficient perimatrix for analysis. In the retro-auricular skin sections there was no evidence of inflammation.

Immunohistochemical staining of Ki-67

In the retro-auricular skin, the proportion of Ki-67-positive cells in the upper basal layer ranged from 2.0% to 13.0%. The cholesteatoma epithelium showed positive Ki-67 staining in cells of the upper basal layer and to a lesser extend in the suprabasal layers (Fig1B). In the connective tissue some Ki-67-positive cells were present. The proportion of Ki-67-positive nuclei in the cholesteatoma epithelium showed large individual variations, ranging from 1.7% to 35.6%. On average, however, the cholesteatoma samples showed a significantly increased percentage of Ki-67-positive cells, compared to retro-auricular skin ($p=0.031$). The average percentages of Ki-67-positive cells in retro-auricular skin and cholesteatoma epithelium are summarized in Table1. The increased cell proliferation in the cholesteatoma epithelium was not related to the presence of inflammation, as non-inflamed cholesteatoma tissues also showed an increased Ki-67 expression.

Tissue	Ki-67	p53	p21
Retro-auricular skin	5.7 ± 3.6	5.8 ± 5.4	1.0 ± 0.9
Cholesteatoma epithelium	11.7 ± 9.5*	17.8 ± 12.3**	14.3 ± 11.6**

Table 1. *Percentages of Ki-67-, P53- and P21-positive cells in control skin and cholesteatoma epithelium.**p< 0.05; **p< 0.01 versus retro-auricular skin.

Immunohistochemical staining of p53

The retro-auricular skin samples showed 0.8% to 19.2% p53-positive cells in the basal layer. Most of the staining for p53 was found in the cells of the basal layer of the cholesteatoma epithelium, with some staining in cells in the connective tissue (Fig.1C.). The proportion of p53-positive cells in the cholesteatoma epithelium varied greatly, ranging from 0.3% to 39.1%. The percentage of p53-positive cells was significantly increased compared to that in retro-auricular skin ($p=0.007$). The average percentages of p53-positive cells in retro-auricular skin and cholesteatoma epithelium are listed in Table2. We found no difference in p53 expression between inflamed cholesteatoma samples and non-inflamed cholesteatoma samples.

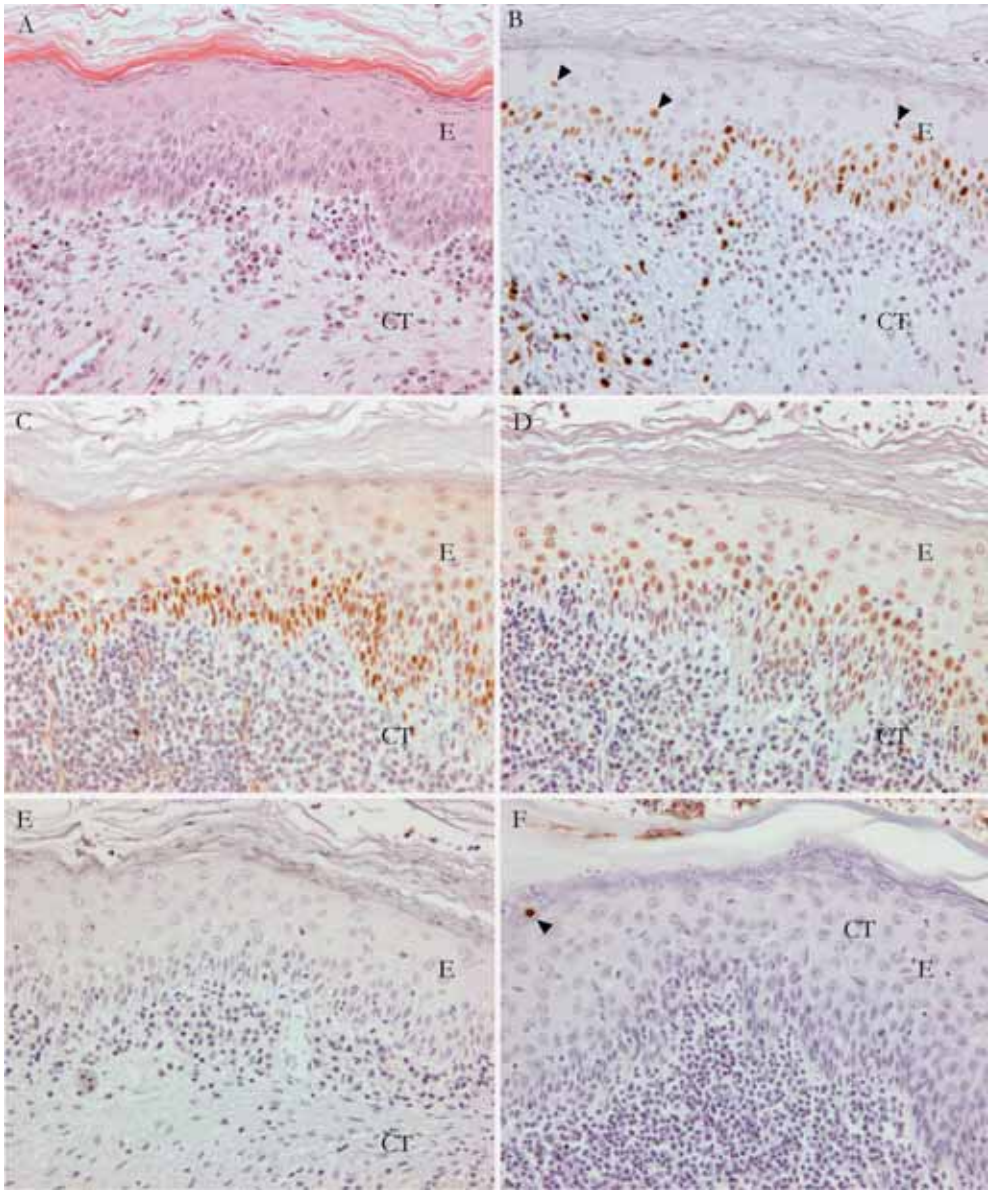

Fig. 1 Immunohistochemical localization of p53, p21 and Ki-67 in serial sections of human cholesteatoma epithelium: (A)HE staining; (B) Ki-67-positive cells are expressed in the basal and suprabasal layers of the epithelium (*arrowheads*); (C) Many p53-positive cells are expressed in the basal layer;(D) p21-positive cells are expressed in the lower suprabasal layers; (E) active caspase3; (F) TUNEL staining showing a positive cell (*arrowhead*). Original magnification x 200. E= epithelium; CT= connective tissue

Immunohistochemical staining of p21

In retro-auricular skin a maximum of 2.1% of the cells were positive for p21. The p21- positive cells of the retro-auricular skin and the cholesteatoma epithelium were locally expressed in the lower suprabasal layers. In some cholesteatoma, p21 expression was also observed in the connective tissue (Fig.1D). p21-positive cells were present in the epithelium of all 15 cholesteatoma samples. The proportion of p21-positive cells varied considerably, ranging from 0.4% to 32.5%. When compared to retro-auricular skin, the percentage of p21-positive cells in cholesteatoma tissue was significantly increased ($p=0.001$). Table 2 lists the percentage of p21-positive cells in retro-auricular skin and cholesteatoma epithelium. We found no difference in p21 expression between samples with or without inflammation.

Co-expression of Ki-67, p53 and p21

Using the Pearson two-tailed correlation test, a significant positive association was observed between p53 and p21 protein expression ($p= 0.016$). We did not find a correlation between any of the other proteins.

Detection of apoptosis

Immunohistochemical staining of active caspase 3

The retro- auricular skin sometimes contained a single positive cell in the granular layer. In the cholesteatoma epithelium samples there were hardly any active caspase 3 positive cells (Fig.1E). In those cells that were positive for active caspase 3, the nuclei showed a fragmented morphology. The data were not used for further calculations as all tissue samples contained a negligible number of positive cells.

TUNEL (modified method)

The retro- auricular skin cells of the granular layer were only occasionally TUNEL-positive. The stromal portion showed a small number of TUNEL-positive cells in 10 cholesteatoma samples. In the stroma of some cholesteatoma samples a few clusters of TUNEL-positive cells were detected, indicating an increase in apoptosis. In some epithelia of cholesteatoma samples a single TUNEL-positive cell was observed (Fig.1F). All TUNEL-positive cells had fragmented nuclei. The samples showed a negligible number of TUNEL-positive cells and therefore these data were not used for further calculations.

Discussion

This is the first report to document, in the same study, cell proliferation, cell cycle arrest and apoptosis in cholesteatoma epithelium. In most cholesteatoma samples we observed active inflammation, involving neutrophils, monocytes, macrophages and newly formed blood vessels. In the epithelial layer of the cholesteatoma we found increased expression of the proliferation marker Ki-67. Concomitantly, the cholesteatoma epithelium showed overexpression of p53. The increased Ki-67 expression was not related to the overexpression of p53. In addition, we found a remarkable increased proportion of p21 in the epithelial layer. We demonstrated a significant positive correlation between p53 and p21. Apoptosis, which is determined

by active caspase 3 expression, was not detected in the cholesteatoma epithelium. This was confirmed by the observation that a negligible proportion of apoptotic cells was determined using the TUNEL technique. The abundant presence of the proliferation marker Ki-67 and the overexpression of p53 in cholesteatomal tissue have been reported before[1]. High expression of p53 has also been mentioned by others, but with a subsequent high level of apoptosis[9], as determined using the TUNEL assay. Our analyses, however, clearly demonstrate that there was no increase in apoptosis in the cholesteatoma samples. Our study demonstrates cell proliferation, characterized by increased Ki-67 protein expression, but also an increase in p53 protein level, which suggests a halt to proliferation by means of G1 blockade[7]. These unexpected results might be caused by a dysfunctioning p53 protein. p21, however, is upregulated in cholesteatoma, indicating a transcriptionally active p53 protein[16]. In addition, we have demonstrated that apoptosis in cholesteatoma can be adequately blocked. This is only possible if there is a combination of normal functioning p53 and p21 proteins[17]. Such a concept is also supported by previous reports showing that cholesteatomas are genetically stable and have a normal DNA content[1]. This indicates that, in cholesteatoma, apoptosis may not be required as a cellular protection mechanism.

Conflicting results have been established in the cholesteatoma epithelium. The presence of normal functioning p53 and p21 proteins suggests an effective proliferation block. This, however, is not the case. The apparently ineffective proliferation block might be caused by different functions of p21[18]. The p21 molecule initiates the cell cycle arrest by binding to the regulators of the cell cycle, the cyclin/cyclin-dependent kinase (CDK) proteins. It has been demonstrated that the CDK inhibitory function of p21 is regulated stoichiometrically, i.e. only when p21 is in molar excess[19]. In the cholesteatoma epithelium, it is possible that inhibition of CDK activity occurs in cells with a molar excess of p21. When the molar abundance of p21 decreases, because of the short half-life of the p21 molecule[20], the cell cycle is no longer inhibited. This assumption is supported by the observation of suprabasal expression of Ki-67 in the cholesteatoma epithelium[1,2] (Fig 1B.).

The p21 protein itself, however, can act as a positive modulator of cell cycle progression. It has recently been found that, in the presence of calcium, p21 can bind directly to Calmodulin[18]. This interaction appears to be required for nuclear localization of cyclinD/CDK4[21], which switches on proliferation. Most cholesteatoma tissues exhibit a considerable bone resorption. Therefore, high local concentrations of calcium might be present. Under these specific conditions, the p21 protein can initiate cell proliferation in the cholesteatoma. Inflammation or the presence of endotoxins can also trigger proliferation. We therefore assume that, in the epithelial layer of the cholesteatoma, the Ki-67 protein is increased by means of different signals. This is also plausible for the upregulation of the p53 protein. Hyperproliferation can increase p53, but neutrophils can also activate the p53 protein by releasing reactive oxygen. It appears that, in cholesteatoma epithelium, the coordination of the cell cycle has become dysfunctional. However, our results prove that the p53 protein can effectively upregulate p21 expression as protection against apoptosis. In this study we have demonstrated that increased proliferation

in the cholesteatoma is not compensated by apoptosis, but may be associated with cell cycle arrest. Future research will focuss on the role of p21 in stem cell commitment and differentiation in the cholesteatoma.

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Chapter 4

4

Sustained extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase signalling is related to increased p21 expression in cholesteatoma epithelium.

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Abstract

Conclusion. These results show for the first time that the RAS/ RAF/ ERK1/2 MAPK signalling pathway is active and involved in p21-mediated cell cycle arrest in human cholesteatoma epithelium.

Objective. In a previous report we have demonstrated that the epithelium in human cholesteatoma, is characterized by high p53-dependent p21 expression. The RAS/ RAF/ extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) signalling pathway can induce p21 expression and subsequent cell cycle arrest via p53-dependent or -independent mechanisms.
We designed the present study to investigate whether the RAS/ RAF/ ERK1/2 MAPK signalling pathway is involved in p53-dependent and p21-mediated cell cycle arrest in human cholesteatoma.

Material and methods. A total of 18 cholesteatoma samples and 18 paired control retro-auricular skin samples were immunohistochemically stained for p53, p21, phosphorylated ERK1/2 (pERK1/2) and total ERK1/2. Positive cells were counted by means of digital image analysis. Double-label fluorescence immunohistochemistry was performed to demonstrate co-expression of p21 and pERK1/2.

Results. Protein expression of p53, p21 and pERK1/2 differed significantly between cholesteatoma epithelium and retro-auricular skin ($p < 0.01$). In cholesteatoma, co-expression of p21 and pERK1/2 was prominent, whereas in retro-auricular skin there was hardly any co-expression. Positive correlations were found between p53 and p21 ($p = 0.003$) and between p21 and pERK1/2 ($p = 0.013$).

Keywords: *Cell cycle arrest, cell signalling, immunohistochemistry, p53*

Introduction

Cholesteatoma is a non-malignant, destructive ear disease, caused by the presence within the middle ear cleft of keratinizing stratified squamous epithelium. Several theories of the pathogenesis of human cholesteatoma have been described, the most important being the migration, invasion and proliferation theory[1]. The link between these theories is disruption of, or damage to, the lamina propria of the tympanic membrane, which usually occurs in combination with a chronic middle ear infection. The biological properties and inductive forces of the inflammation may lead to the invasion of stratified squamous epithelium into the middle ear cleft, causing the formation of cholesteatoma. Clinical sequela may include destruction of the middle ear ossicles and other structures. Cholesteatoma epithelium deviates from normal epidermis in terms of hyperplasia, aberrant differentiation and progressive accumulation of keratins[2]. In human epidermis, normal turnover of keratinocytes depends on controlled coordination between proliferation and differentiation. This requires a regular supply of de novo differentiated cells. It has been demonstrated that irreversible cell cycle arrest is an early and integral part of epidermal differentiation[3]. A key protein involved in cell cycle arrest is the cyclin-dependent kinase inhibitor (CDKI) $p21^{cip1/waf1.}$ Binding

of the p21cip1/waf1 protein to cyclins and cyclin-dependent kinases (CDKs) prevents transition of the cell cycle from the G1 phase to the S phase and from the G2 phase to the M phase[4]. P21cip1/waf1 also inhibits DNA replication by association between the carboxy-terminal domain of p21cip1/waf1 and proliferating cell nuclear antigen (PCNA). At least two DNA- metabolizing enzymes, i.e. Fen1 and DNA (cytocine-5)methyltransferase, have been shown to bind to the same overlapping region of PCNA in competition with p21[5]. Association with cyclins, CDK, and PCNA makes the p21 cip1/waf1 protein an essential component of cell cycle control. Expression of p21 cip1/waf1 is usually controlled at the transcriptional level by p53-dependent and -independent mechanisms[6]. The mitogen-activated protein kinase (MAPK) pathway RAS/ RAF/extracellular signal- regulated kinase (ERK)1/2 has been described as being both a p53-dependent and -independent mechanism of p21 induction[6-9]. Previously, we have demonstrated that the p21 cip1/waf1 protein expression was increased and positively correlated with p53 expression in human cholesteatoma epithelium, compared to normal skin[10]. In cholesteatoma, Huang et al have also demonstrated an increased expression of the RAS protein[11]. Because of the essential role of p21 in cell cycle control, we investigated whether the RAS/ RAF/ ERK1/2 MAPK pathway is involved in p53-dependent, p21-mediated cell cycle arrest in human cholesteatoma epithelium. For this purpose we determined the expression of: (i) p53, (ii) p21, (iii) phospho-activated ERK1/2 (pERK1/2) and (iv) total ERK1/2. In addition, co-expression of pERK1/2 and p21 proteins was investigated by means of double-label fluorescence immunohistochemistry using confocal laser scanning microscopy. The results obtained with cholesteatoma tissue were compared to paired control samples from retro-auricular skin.

Materials and methods

Clinical and histopathological data

Cholesteatoma specimens and biopsies of retro-auricular skin were obtained from 18 patients. The samples were placed in phosphate-buffered saline immediately after the operation. The Committee of Medical Ethics of 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 (4-μm thick) were taken from each tissue block. The first and last sections were stained with hematoxylin eosin. The sections were immunostained with antibodies respectively against p53 (clone DO7), p21waf1/cip1 (clone SX118), phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2. Anti-p53 and anti-p21 were monoclonal antibodies purchased from Dako B.V. (Glostrup, Denmark) and anti-pERK1/2 and anti-total ERK1/2 were polyclonal antibodies obtained from Cell Signaling Technology (Beverly, MA). The dilutions used for anti-p53 and anti-p21were 1: 100, 1: 500 and for anti-pERK1/2 and anti-total ERK1/2,1: 200 respectively. Double staining was performed by means of immunofluorescence. Anti-p21 was conjugated to goat anti-rabbit horseradish peroxidase (HRP; Kirkegaard & Perry Laboratories,

Gaithersburg, MD) and labelled with Tyramid-fluorescein isothiocyanate (FITC; 1:100; kindly provided by Dr. C. van Kooten, Department of Nephrology, Leiden University Medical Center). Anti-pERK1/2 was labelled with goat anti-rabbit Ig conjugated to Alexa 546 (1:100; Molecular Probes, Leiden, The Netherlands). Sections from the same tissue served as negative controls, i.e. the primary antibody was omitted.

Expression of single proteins

Expression of p53, p21, pERK1/2 and total ERK1/2 was determined using an indirect immunoperoxidase method. Sections from sigmoid (p21) and colon carcinoma (p53, pERK1/2 and total ERK1/2) were used as positive controls. After deparaffination, the sections were treated with methanol containing 0.3% hydrogen peroxide for 20 minutes in order to inactivate endogenous peroxidase. The sections were rehydrated and subjected to microwave antigen retrieval in boiling citrate buffer (0.01M, pH 6.0, for 10 min)[12]. All sections were washed in Tris-buffered saline (TBS) and incubated with the primary antibody in 0.2% bovine serum albumin (BSA) in TBS. The sections were incubated overnight at room temperature (RT) and then washed four times in TBS. The sections used for the p21 antibody treatment were subjected to a secondary incubation with rabbit anti-mouse in Dako diluent buffer (1:2000) for 20 min at RT. After washing in TBS, the p21, pERK1/2 and total ERK1/2 sections were incubated with ChemMate Envision (anti-rabbit and anti-mouse; Dako) for 30 min at RT and developed with 3,3'-diaminobenzidin chromogen (DAB+; 1:50; Dako), for 5 min at RT. The sections were counterstained with hematoxylin for 1 min. The other specimens were incubated with the appropriate biotinylated secondary antibody for 30 min at RT, washed with TBS and subsequently incubated with peroxidase-conjugated streptavidin (Dako) at RT for 30 min. They were then treated with DAB chromogen containing 0.02% H_2O_2 for 15 min and counterstained with hematoxylin for 1 min.

Co-expression of p21 and pERK1/2

The sections were deparaffinized and treated with TBS containing 0.09% H_2O_2 and 0.1% sodium azide for 20 min, in order to block endogenous peroxidase. They were then rehydrated and subjected to microwave antigen retrieval in boiling citrate buffer (0.01M, pH 6.0 for 10 min)[12]. After washing in TBS the sections were incubated with both primary antibodies against p21 and pERK1/2 in 0.2% BSA in TBS overnight at RT. They were then washed four times in TBS and incubated with goat anti-mouse Ig-HRP and goat anti-rabbit-Alexa 546 for 30 min at RT. The sections were subsequently incubated with Tyramid-FITC in Tris-HCl buffer (0.2 M Tris-HCl, pH 8.8, 0.1 M imidazole, 0.0001% H_2O_2) for 30 min at RT. After washing in TBS, the sections were mounted in Vectashield (Brunschwig Chemie, Amsterdam, The Netherlands). The cross-reactive background of all reagents could be excluded by sequential omission of individual reagents, resulting in either single labelling or absence of labelling.

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, UK.). 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 positive immunohistochemical staining has been described elsewhere[13]. For each section, images from at least five different areas were stored as digitised images. For cell counting the same areas of the sections were used , but with various stains. 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.
Confocal laser scanning microscopy was performed using a confocal laser scanning microscope (Zeiss LSM510) in a multi-track setting. FITC was excited at 488nm and detected using a 505-530 band-pass filter. Alexa-546 was excited at 543nm and detected using a 560-615nm band-pass filter. Using these settings the two fluorochromes could be detected separately without any background in the other channels. Each fluorochrome was given an artificial colour: FITC, green; Alexa-546, red. A PH2 Plan-NEOFluar 25 x 0.80 Imm Korr objective was used.

Data analysis
Data are expressed as mean ± 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 expression in cholesteatoma and retro-auricular skin. The level of significance was set at $p < 0.05$. The Spearman's rank correlation test was used to calculate correlations. Correlations were considered significant at the 0.05 level. The SPSS10 software package (SPSS, Chicago, IL) was used for the calculations.

Results

Histopathological findings
On macroscopic inspection at surgery 11/18 cholesteatoma were considered clinically infected. In13/18 cholesteatoma samples, microscopic inspection revealed inflammatory cells and newly formed blood vessels in the connective tissue. In three tissue samples there was insufficient connective tissue for analysis. In the retro-auricular skin sections there was no evidence of inflammation.

Expression of p53, p21, pErk1/2 and total ERK1/2
Positive expression of p53 was mainly found in the cells of the basal layer but also in the cells of the suprabasal layers of the cholesteatoma epithelium (Figure 1A). The percentage of p53-positive cells was significantly increased compared to that of retro-auricular skin ($p = 0.003$). The average percentages of p53-positive cells in retro-auricular skin and cholesteatoma epithelium are listed in Table I. p21-positive cells were present in the epithelium of all 20 cholesteatoma samples,. p21-positive cells were locally expressed in the lower suprabasal layers of the

retro-auricular skin and cholesteatoma epithelium (Figure 1B). In cholesteatoma, p21 expression was also observed in the upper suprabasal layers. Compared to retro-auricular skin, the percentage of p21-positive cells in cholesteatoma tissue was significantly increased ($p < 0.001$). Table I lists the percentages of p21-positive cells in retro-auricular skin and cholesteatoma epithelium. Expression of pERK1/2 in cholesteatoma epithelium was mainly nuclear and localized in all layers of the cholesteatoma epithelium. The location of total ERK1/2 in the epithelial tissue was similar, but total ERK1/2 expression was also more cytoplasmatic (Figures 1C and D). These observations were consistent with the expression of pERK1/2 and total ERK1/2 in retro-auricular skin. Total ERK1/2 staining was performed as a control of the location of pERK1/2 and for that reason the cells were not counted. On average, cholesteatoma samples showed a significantly increased percentage of pERK1/2-positive cells compared to retro-auricular skin ($p<0.001$). The average percentages of pErk1/2-positive cells in retro-auricular skin and cholesteatoma epithelium are summarised in Table I.

Associations of p53, p21 and pErk1/2 with inflammation

We investigated whether there was a correlation between the samples which were considered clinically infected and protein expressions, but could not find no such relationship. We also found no relationship between the presence of inflammatory cells and protein expressions.

Associations of p53, p21 and pErk1/2

Using Spearman's rank correlation test, a significant positive association was observed between p53 protein expression and p21 ($p=0.003$) and between p21 protein expression and pErk1/2 ($p=0.013$). We found no correlation between p53 and pErk1/2.

Co-expression of p21 and pErk1/2

In cholesteatoma epithelium, confocal laser scanning microscopy revealed that almost all cells in the basal cell layer were pERK1/2 positive. In this layer hardly any ERK1/2 positive cell showed p21 co-expression. In the suprabasal layers co-expression of pERK1/2 and p21 was prominent. (Figures 1E-H). In retro-auricular skin we found almost no co-expression.

Tissue	p53	p21	pERK1/2
Retro-auricular skin	1.7 ± 1.9	1.9 ± 1.4	0.8 ± 0.7
range	0.2 to 4.6	0.1 to 3.4	0.1 to 2.8
Cholesteatoma epithelium	6.3 ± 4.6	11.1 ± 9.4	10.0 ± 7.5
range	0.8 to 13.7	1.3 to 40.6 .	0.2 to 26.9

Table 1. Percentages of P53, P21and pERK1/2-positive cells in control skin and cholesteatoma epithelium.

Fig. 1

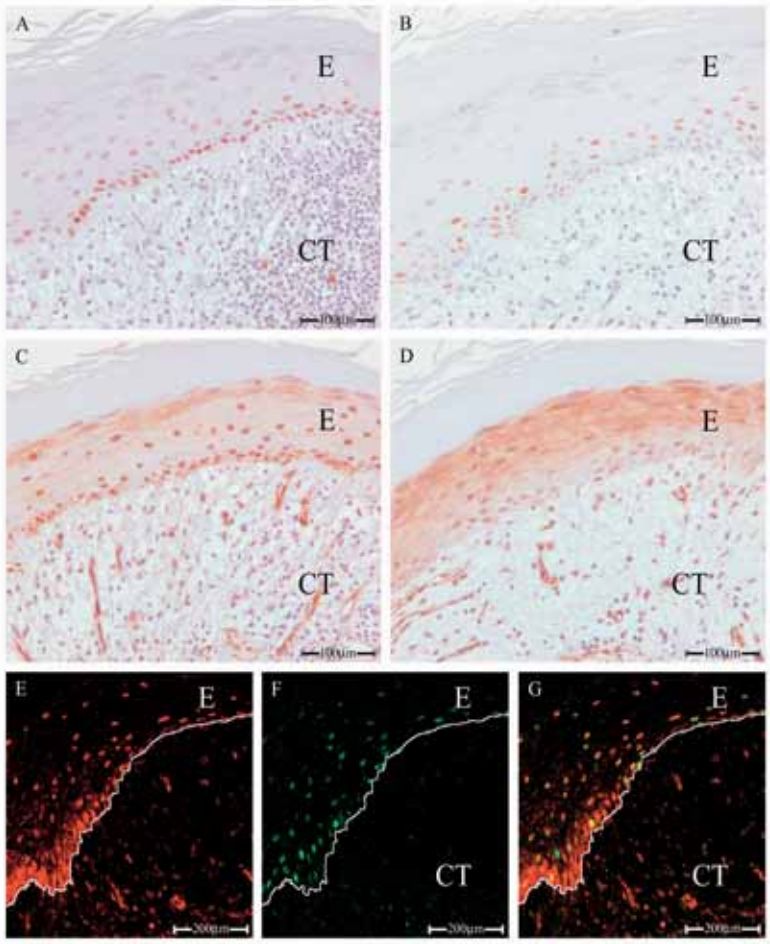

Figure 1. Immunohistochemical localization of P53, p21 and Ki-67 in serial sections of human cholesteatoma epithelium: (A) HE staining; (B) Ki-67-positive cells are expressed in the basal and suprabasal layers of the epithelium (*arrowheads*); (C) Many p53- positive cells are expressed in the basal layer; (D) p21-positive cells are expressed in the lower suprabasal layers; (E) active caspase3; (F) TUNEL staining showing a positive cell (*arrowhead*). Original magnification x 200.
E = epithelium; CT= connective tissue

Discussion

Our results show that in human cholesteatoma epithelium the RAS/ RAF/ ERK1/2 MAPK pathway is involved in p53-dependent increased expression of p21. This is demonstrated by the correlated expression of p53 and p21 and of p21 and active ERK1/2. It has been demonstrated that the RAS/ RAF/ ERK1/2 MAPK signalling pathway is activated in response to many mitogens, such as growth factors, cytokines, bacterial endotoxins, insulin and osmotic stress[14]. It has also been reported that activated RAS elicits premature cell senescence (G1 arrest), which is accompanied by increased expression of p53, p21 and p16[15]. Although transcription factors are important MAPK targets, MAPK can also regulate protein expression through post-transcriptional mechanisms[16,17]. Differential processes may be of importance in this context. The process of post-transcriptional-regulated stabilization of the p53 protein via RAF activation has been reported to result in increased p21 expression[18]. Another post-transcriptional effect of activated MAPK may be upregulation of the p21 protein by preventing of its degradation by the 26S proteasome complex[17].

Curiously, in the MAPK signalling pathway, ERK1/2 activation has been associated with both stimulation and inhibition of cell proliferation. In this context, it has been shown in several studies that the magnitude of ERK1/2 activation determines whether the cellular response includes the induction of p21[6,9,18]. Thus, the induction of p21 expression requires a stronger ERK1/2 signal than ERK1/2-mediated induction of proliferation. It has been demonstrated that with high and sustained ERK1/2 activity, p21 binding to the proteasome complex is inhibited[18], i.e. the proteasomal degradation of p21 is reduced, which may result in high expression of p21[9,17].

Roper et al. have shown that, for proper RAS/ RAF/ ERK1/2 MAPK-induced cell cycle arrest, both p53 as well as p21, need to be present[19]. Increased stability of p53 after RAF activation may then lead to transcription of p21. Roper et al. observed that this RAF-mediated induction of p21 is lost in $P53^{-/-}$ cells. This group also reported that p53 is not induced at the RNA level after RAF activation. This may indicate that RAF regulates p53 at the level of protein stabilization[19], which has also been confirmed by others[20]. Reciprocal to this, Lee et al. showed that p53 can also mediate MAPK activation[21]. This may imply a positive feedback loop in which permanent growth arrest could be augmented by sufficient upregulation of either p53 or MAPK pathways. Moreover, Lee et al. found that p53-mediated ERK1/2 activation is higher and more sustained than that without p53 involvement[21]. These reports fit well with the expression profile observed in cholesteatoma epithelium. Increased RAS expression has previously been demonstrated in human cholesteatoma epithelium[11] and high expression of activated ERK1/2 implies an active RAS/ RAF/ ERK1/2 MAPK signalling pathway. Furthermore, increased p53 and p21 expression was detectable in all suprabasal layers in cholesteatoma epithelial tissue, indicating an extended lifetime of both proteins. The correlation between p53 and p21 expression may imply a direct involvement of p53 in p21 expression. Finally, the co-expression of pERK1/2 with p21 and their correlation, although less than that between p53 and p21, indicates that in human

cholesteatoma pERK1/2 is also involved in the mechanism of p21 upregulation.
We speculate that, in human cholesteatoma, activation of MAPK is mainly caused by external factors, such as inflammation-induced growth factors, cytokines and bacterial endotoxins. These factors may also be responsible for primary upregulation of p53 with subsequent transcription of p21. Low or intermediate concentrations of pERK1/2 may result in progression through the cell cycle. When triggering is prominent, e.g. as a result of different mitogenic stimuli, strong RAS/ RAF/ERK1/ 2 activation may occur[9]. Stabilization of p53 by RAF may then induce positive feedback to produce a sustained increased level of activated ERK1/2 (Figure 2). The latter factor is a major condition for the prevention of p21 breakdown, which is a prerequisite for proper cell cycle inhibition[9]. In cholesteatoma epithelium, these differential processes were visualised by single pERK1/2 expression in the proliferating compartment. In the suprabasal layers, when pERK1/2 expression was sustained the cells were also found to be positive for p21(Figures 1E-G).
We postulate that, in cholesteatoma cell cycle arrest, posttranscriptional mechanisms such as p53 stabilization and prevention of p21 breakdown, play prominent roles. Prolonged MAPK-induced cell cycle inhibition causes accumulation of epithelial cells, which are in G-phase arrest[18]. In human cholesteatoma, prevalent

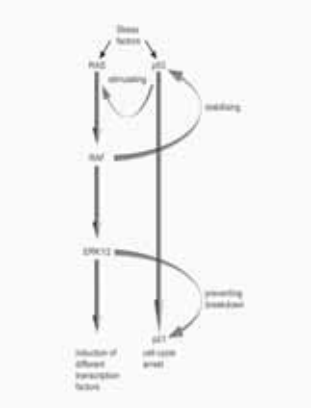

Figure 2. Induction of p21 transcription in human cholesteatoma epithelium. External triggers, like growth factors initiate the activation of the MAPKinase pathway and the transcription of different proteins involved in proliferation. External triggers may also initiate the production of p53. When there are different stimuli, both the MAPKinase pathway and the production of p53 are initiated. RAF activation may then result in stabilisation of p53, which may lead to a further activation of RAS and a sustained ERK1/2 phosphorylation. This prevents p21 breakdown, which is central to an increase in p21 concentration and cell cycle arrest

cell cycle arrest may contribute to epithelial hyperplasia.
MAPK signalling, however, is not only restricted to a special cell type, which led us to presume that different stromal cells are also activated. The study of MAPK signalling in all cholesteatoma cells may, in our opinion, help us in the understanding of processes such as invasion, bone resorption, re- inflammation, etc.
Our next study will concern the specific role of the different mitogenic stimuli in the activation of the RAS/ RAF/ ERK1/2 MAPK pathway in human cholesteatoma epithelium.

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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.

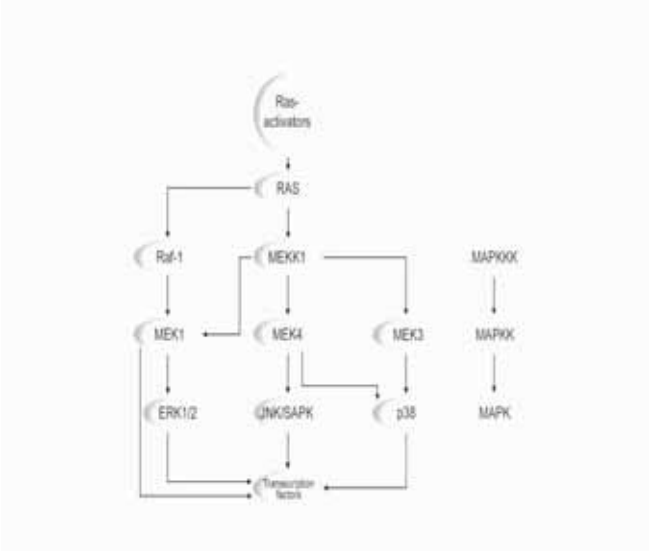

Figure 1. The classic MAPK cascade consists of three sequental intracellular activation steps and is initiated when the first member, MAPKKK, is activated. MAPKKK activates MAPKK. Subsequently, MAPKK activates a MAPK. There are three MAPK pathways downstream including Ras/ Raf/ MEK1/ ERK, Ras/ MEKK1/ 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

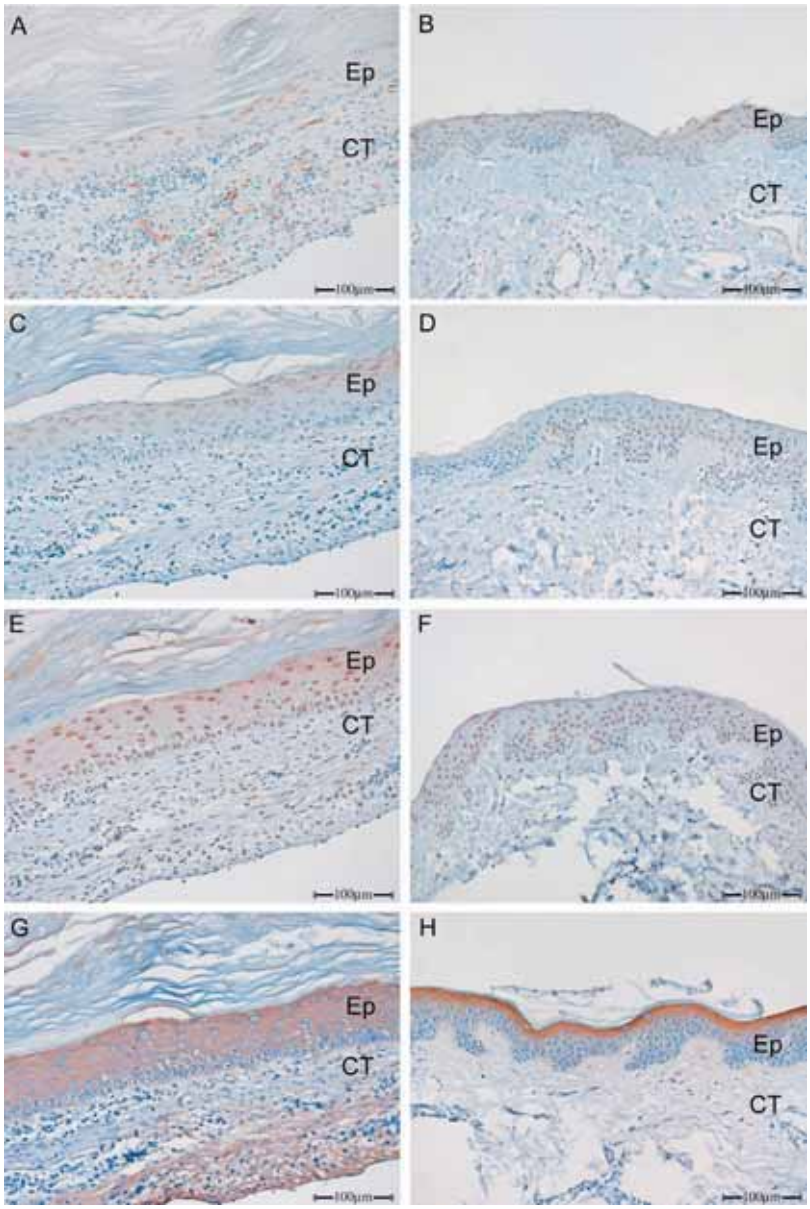

Figure 2. Immunohistochemical localization of pERK1/2, pJNK/SAPK, pp38, and involucrin in paraffin sections of human cholesteatoma epithelium and paired retro-auricular skin; (A and B) pERK1/2-positive cells are expressed in all epithelial layers; (C and D) pJNK/SAPK-positive cells are expressed in all 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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Chapter 6

Survival signalling and terminal differentiation in cholesteatoma epithelium.

6

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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 reaction[1]. 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 cleft[2]. 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 outcome[3] 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 results[6]. 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 survival[7]. 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 inflammation[11]. 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 filaggrin[14]. 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 marker[15]. In cholesteatoma epithelium, besides minimal apoptosis, we have shown ERK1/2 and p38 MAPK activation, to be associated with terminal differentiation[6]. 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 ìm 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 elsewhere[17]. 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 ± 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 to100.0	41.0 to 93.5	72.0 to96.4	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.
* $p<0.001$; ** p0.003 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-aptotic 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 filaggrin[14]. 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 increased[19]. 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 reported [20-22]. In one of these reports it has been mentioned that augmented apoptosis explains the diminished presence of late terminal differentiation markers in psoriasis[21]. In that study the TUNEL assay has been used but in another study DNA analysis revealed that psoriatic keratinocytes have intact DNA[23]. Metze et al suggested that abnormalities of cell surface adhesion structures might account for the dysregulation of the cornified envelope components[22]. 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 complexes[14]. 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[24]. Their proposal that matrix metalloproteases (MMPs) play a prominent role in this process may hold true, because MMP activity has been frequently reported in cholesteatoma[25].
MAPK activation has been reported to generate early terminal differentiation by promoting involucrin transcription[15]. Interestingly, sustained ERK1/2 MAPK activation is required for anchorage-independent survival of epithelial cells[10].
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 diseases[22]. The defect in the production of filaggrin may result in a failure to degrade keratohyalin granules[22]. This may explain the conspicuous granular appearance of the cholesteatoma cornified layer [1]. 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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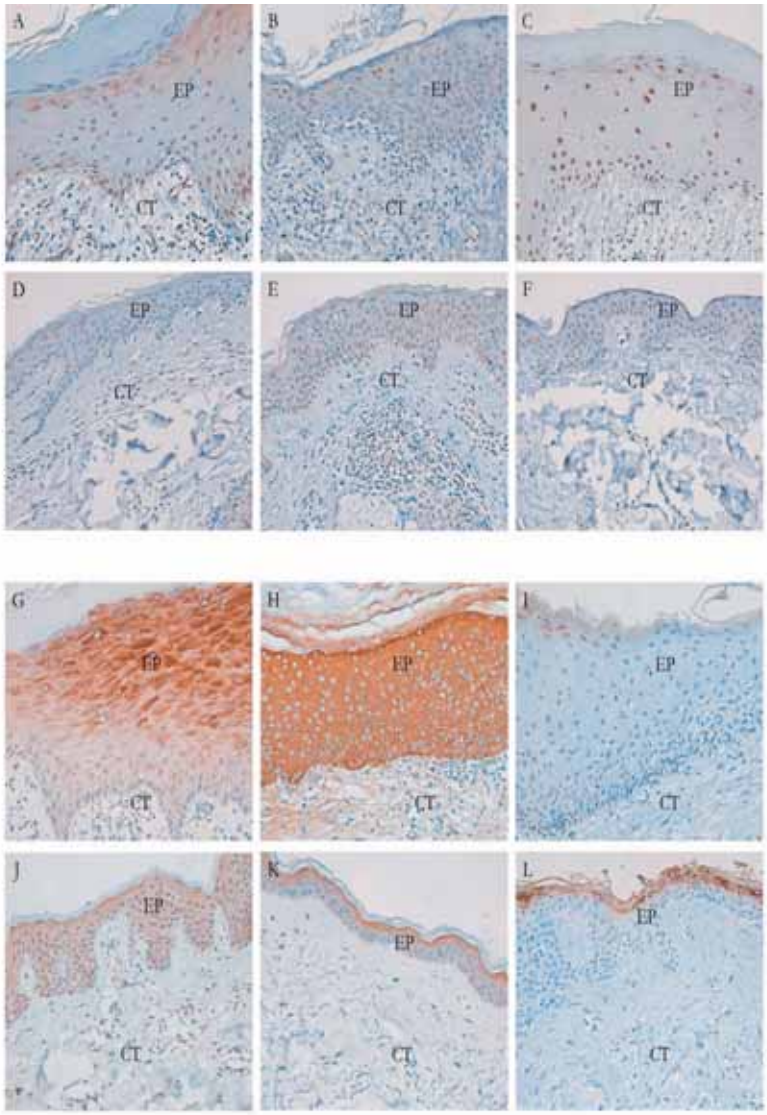

Figure 1.
Immunohistochemical analysis/ staining of paraffin sections of human cholesteatoma epithelium (A,B,C,G,H,I) and retro-auricular skin(D,E,F,J,K,L). Localization of p ERK1/2(A,D), pJNK/SAPK(B,E), pp38(C,F), pAkt(G,J), involucrin(H,K) and filaggrin(I,L) in Original magnification: x 200.

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Chapter 7

Human cholesteatoma behaves as a chronic wound: the role of transforming growth factor β

7

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Submitted

Abstract
Cholesteatoma is a non-malignant, destructive lesion of the temporal bone that gradually expands and causes complications by erosion of the adjacent bony structures. The consequences can be as severe as facial paralysis and intracranial complications. Until now, surgery is the only treatment of choice. The pathogenesis of cholesteatoma remains still controversial. Current concepts postulate that cholesteatoma may be considered a wound healing process, although formal proof is lacking as yet. Several reports provide evidence for the involvement of TGFβ in both normal and abnormal wound healing. In the present study, quantitative immunohistochemical analysis was performed to examine the expression of TGFβ, the activated form of its intracellular effector, phosphorylated-Smad2 (pSmad2), its natural inhibitor Smad7 and target gene EDA-positive fibronectin (EDA-FN). In 12 cholesteatoma and control samples protein expressions showed consistent relationships among TGFβ, nuclear pSmad2 and Smad7. We found concordant expressions of TGFβ and nuclear p-Smad2 in cholesteatoma epithelium and its control. Epithelial Smad7 expression was significantly reduced in cholesteatoma when compared to control epithelium ($p=0.04$). In cholesteatoma extracellular matrix (ECM), a significantly increased TGFβ and nuclear pSmad2 was demonstrated ($p<0.01$). Smad7 expression in the ECM was comparable in cholesteatoma and its control. EDA-FN deposition in cholesteatoma ECM was excessive whilst EDA-FN expression was absent in controls. Our results confirm reports of in vitro experiments and support the concept that cholesteatoma behaves like a chronic wound healing process.
Keywords: pSmad2, Smad7, Fibronectin EDA, wound healing.

Introduction
Cholesteatoma has been described since the 17th century and has been the subject of extensive research. Two main types of cholesteatoma have been described: congenital, which occurs behind an intact tympanic membrane, and acquired. Acquired cholesteatoma can appear as a limited diverticulum of the pars flaccida but also with posterosuperior eardrum perforations[1]. This type is usually associated with inflammatory reactions in the middle ear cavity and in active cholesteatoma inflammatory granulation tissue often appears along the invading epithelium[1]. Although the origin of cholesteatoma is not clear, the migration theory, in which keratinocytes from the external ear canal migrated into the middle ear cavity, forming a pathological collision with middle ear mucosa is generally accepted. As a result of this collision, cholesteatoma epithelium and stroma are entangled (Fig. 1), which makes separate analysis virtually impossible.
There is still a difference of opinion concerning cholesteatoma pathogenesis, and different hypotheses have been proposed. Among current concepts it is postulated that cholesteatoma can be considered as disturbed wound healing[2]. 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[2]. Wound healing is a complex process, which involves a series of overlapping stages subdivided in inflammation, granulation formation, extracellular

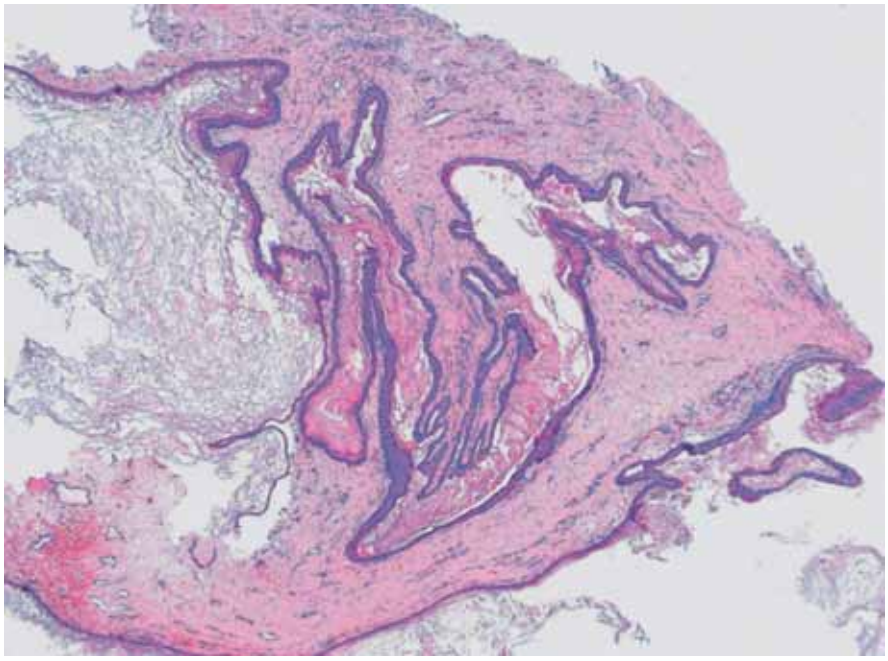

Figure 1. Hematoxylin-eosin staining of cholesteatoma. Clearly visible is the entanglement between epithelium and connective tissue.

matrix production as well as tissue remodeling[3]. When this delicate balance is disturbed in skin tissue, chronic fibrosis and keloid-formation can be the result. Many reports provided evidence that TGFβ is one of the key factors involved in wound healing[3,4]. TGFβ is able to affect proliferation and migration of different cell types[5,6]. Moreover, TGFβ controls both the initiation and resolution of inflammatory responses by chemotaxis, activation, survival and apoptosis of different inflammatory cells such as lymphocytes, natural killer cells and macrophages[7]. TGFβ is also able to bind to its own gene promoter resulting in amplified biosynthesis[4]. Proper regulation of TGFβ signaling is therefore essential in normal tissue repair. TGFβ signal transduction is activated following ligand binding to the type II TGFβ receptor and, after heteromerization and transphosphorylation of the type I TGFβ receptor, signal propagation occurs by phosphorylation of the receptor-specific (R) Smads (Smad2 and Smad3). The phosphorylated R-Smads then oligomerise with Smad4, translocate to the nucleus and regulate the transcription of target genes[8]. The TGFβ downstream signaling can be inhibited by an antagonist of the Smad-family, Smad7[8]. Association of different Smads and Smad complexes with transcription factors and transcriptional co-activators/co-repressors in the nucleus further regulates transcriptional control by TGFβ[8]. It is recognised that after TGFβ-ligand binding to the receptor different other cellular pathways can be stimulated[9]. Moreover, the pathways that are stimulated are thought to be cell type specific[9]. In keratinocytes, TGFβ induces cell cycle arrest

through the induction of $p21^{cip1/waf1}$ transcription[10]. In fibroblasts, however, TGFβ signaling induces proliferation, and as a result of this, collagen production[4]. In cholesteatoma tissue, because of the presence of many different types of cells in the epithelium and stroma, bioactivation of TGFβ is rather complex. To investigate TGFβ bio activation in cholesteatoma, we established TGFβ-induced downstream signaling in both, cholesteatoma epithelium and extracellular matrix. We therefore examined the expression patterns of TGFβ, its activated downstream effector phosphorylated-Smad2 (pSmad2) and the TGFβ signaling antagonist Smad7. Bio activating of TGFβ has been shown to result in alternative splicing of fibronectin transcripts[11]. The EDA^+ FN, isoform is usually seen in fetal and tumor cells and during wound healing but not in normal adult cells[12,13]. Inclusion of the EDA region has also been reported to be essential for normal wound healing[14], although accumulation of EDA-positive fibronectin protein can also be present in fibrotic lesions[15]. For these reasons we focussed our analysis on the expression of this extracellular matrix isoform as a marker for TGFβ bioactivation.

Materials and Methods

Clinical and histopathological data

Cholesteatoma specimens and biopsies of retro-auricular skin were obtained from twelve patients. The Committee of Medical Ethics of the Leiden University Medical Center had approved the protocol. The specimens were prepared for histological examination by fixation in 4% buffered formaldehyde for 20 hours, dehydration in ethanol and embedding in paraffin wax.

Immunohistochemistry

The various protein expressions were visualized with an indirect immunoperoxidase technique. We applied the following antibodies: TGFβ1(V), -pSmad2, -Smad7 and -fibronectin EDA (IST-9). The dilutions used were 1: 50, 1:200, 1: 600 and 1:100 resp. The secondary antibody was ChemMate Envision (anti-rabbit, anti-mouse DAKO, Glostrup, Denmark). Anti-TGF-β1 and fibronectin EDA were purchased from respectively Santa Cruz Biotechnology, Inc.CA USA and Abcam Cambridge, UK. The protein A affinity-purified pSmad2 and Smad7 rabbit polyclonal antibodies have been published before[16,17]. Kidney sections with diabetic nephropathy 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 methanol containing 3% hydrogenperoxide for 20 minutes. After rehydration, the sections were subjected to microwave antigen retrieval in citrate buffer (0.01M, pH 6.0, 12 min.)[18]. All sections were incubated with the primary antibody overnight at 4ºC. and washed in Tris-buffered saline (TBS). The specimens were incubated with the secondary antibody for 30 min. at room temperature (RT), washed and subsequently incubated with peroxidase-conjugated streptavidin at RT for 30 min. They were then treated with 3,3´, di- aminobenzidine chromogen (DAB+, 1:50, 5 minutes at RT; DAKO,

Glostrup, Denmark) and counterstained with hematoxylin for 1 min.

Morphometric analysis of immunohistochemical data

For each of the immunohistochemical markers studied, the DAB positive staining was quantified using an image analysis system (Leica Microsystems Imaging Solutions Ltd. Cambridge, UK.). The microscope was a Leica DMLB with a Leica DC 200 digital camera. The computer-assisted system used to determine the immunohistochemical positive staining is described elsewhere[19]. From each section, images of at least five different epithelial and adjacent subepithelial areas were stored as digitized images. Cholesteatoma and retro-auricular skin samples were included when sufficient stroma, i.e., ~25,000 μm^2, was present. For cell counting purposes, the same areas of the sections, but with various stains, were used. The epithelial compartment was delineated on the screen and the positive and negative cells were counted automatically. In each section more than one thousand cells were counted, and the percentage of positive cells was determined.

Data analysis

Data values were expressed as means ± SD. Because of the non-parametric distribution of the data, the non-parametric Wilcoxon test was used to compare means of the paired variables, with $p < 0.05$ was considered to be significant. To calculate possible correlations, the Spearman's test for non-parametric correlations was used. Correlation was considered significant at the 0.05 level. The Statistical Package for the Social Science (SPSS10, Chicago, IL, USA) was used for the calculations.

Results

Histopathological findings

At macroscopic and microscopic inspection all cholesteatoma were considered to be inflamed. Microscopic inspection revealed in all cholesteatoma samples inflammatory cells and newly formed blood vessels in the connective tissue. In the retro-auricular skin sections there was no evidence of inflammation.

Epithelial expression of TGFβ, pSmad2 and Smad7.

In cholesteatoma epithelium and control skin, a predominant nuclear staining for TGFβ and pSmad2 and a mainly cytoplasmic Smad7 staining was observed (Fig.2A-F). In retro-auricular skin we sometimes also found nuclear Smad7 staining. TGFβ-, pSmad2- and Smad7- positive cells were found in all layers of cholesteatoma and control skin (Fig.2B, D, F). In cholesteatoma, Smad7 expression appears to be less prominent in the basal layers when compared to the control (Fig. 2E,F). The percentages of TGFβ and pSmad2-positive cells in cholesteatoma were similar to those of retro-auricular skin (Fig.3). The percentage of Smad7 positive cells in cholesteatoma epithelium was significantly lower when compared to retro-auricular skin (Fig.3). In cholesteatoma, a positive correlation was established between TGFβ and Smad7 and between pSmad2 and Smad7 (Table1). In retro-auricular skin, we found no correlations.

Stromal expression of TGF ß, pSmad2, Smad7 and fibronectin EDA.

In cholesteatoma stroma, the percentages of TGFβ and pSmad2 were significantly higher when compared to retro-auricular skin (Fig.4). There was no difference in

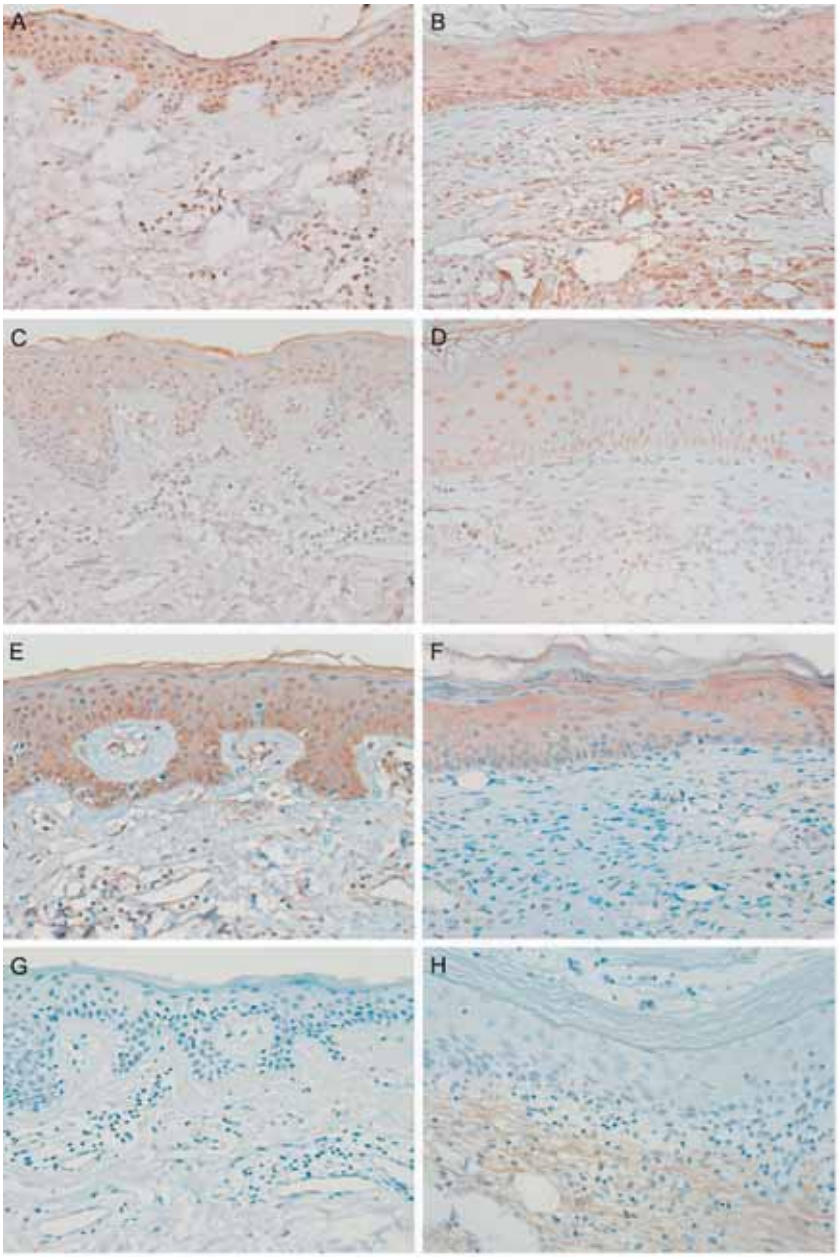

Figure 2. Immunohistochemical localization of TGFβ, pSmad2, Smad7 and fibronectin EDA in paraffin sections of control retro-auricular skin (A, C, E, G) and human cholesteatoma (B, D, F, H). TGFβ-positive cells are prominently expressed in both, epithelium and stroma (A,B). pSmad2-positive cells scatter through the epithelial cell layers and the stroma (C,D). Smad7 is expressed in various epithelial and stromal cells (E,F). Fibronectin EDA-positivity is only present in cholesteatoma stroma (G,H). Original magnification: x 200 E= epithelium; CT= Connective Tissue

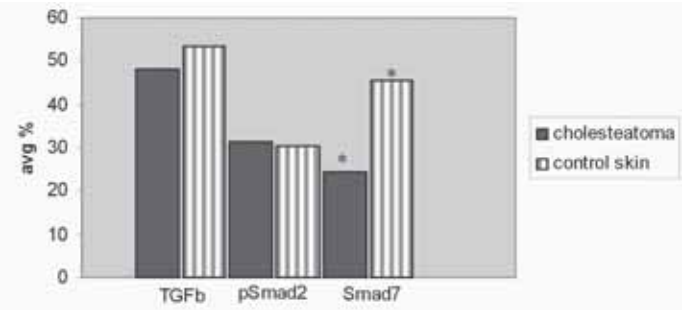

Figure 3. Represents average percentages of TGFβ, pSmad2 and Smad7 in cholesteatoma epithelium and control skin.

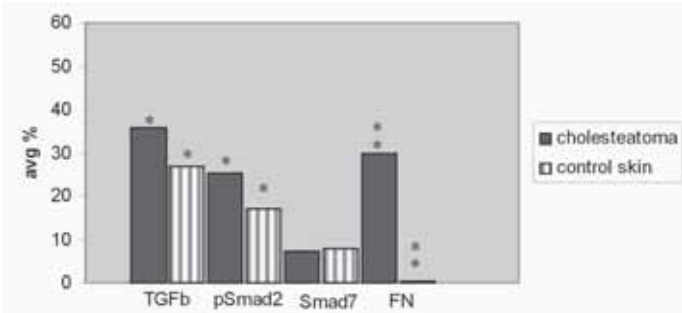

Figure 4. Represents average percentages of TGFβ, pSmad2, Smad7 and fibronectin EDA in cholesteatoma stroma and the stroma of control skin.
*= p<0.05; **= p<0.001.

Cholesteatoma epithelium			
	TGFbeta	pSmad2	Smad7
TGFbeta	-	0.098	**0.021**
pSmad2	0.098	-	**0.003**
Smad7	**0.021**	**0.003**	-

Table 1, representing correlations (p-values) of TGFβ, pSmad2 and Smad7 in cholesteatoma epithelium. Numbers in bold represent significant correlations.

Cholesteatoma stroma			
	TGFbeta	pSmad2	Smad7
TGFbeta	-	**0.001**	**0.039**
pSmad2	**0.001**	-	**0.000**
Smad7	**0.039**	**0.000**	-

Table 2, representing correlations (p-values) of TGFβ, pSmad2 and Smad7 in cholesteatoma stroma. Numbers in bold represent significant correlations

stromal expression of Smad7 in cholesteatoma and that in the stroma of retro-auricular skin. Fibronectin EDA in cholesteatoma stroma was abundantly expressed, while in retro- auricular skin fibronectin EDA expression was absent (Fig 2G,H). In cholesteatoma stroma, TGFβ, Smad2 and Smad7 showed significant positive correlations (Table2). There were no correlations in stroma of retro-auricular skin.

Discussion

Cholesteatoma epithelium

Our data indicate that TGFβ bio-activation appears to be not upregulated in cholesteatoma epithelium. Nevertheless, the correlations among TGFβ, pSmad2, and Smad7 demonstrate that TGFβ signaling pathways are operational. However, in spite of the decreased Smad7 expression these processes do not lead to a significant nuclear pSmad2 expression. A decreased Smad7 expression has previously been demonstrated in scleroderma skin, but in this tissue this was followed by an enhancement in phosphorylation of Smad2[20]. In scleroderma, Smad7 expression appears to be virtually absent while in cholesteatoma epithelium, although decreased when compared to control skin, the average Smad7 positivity was 20%. The competitive binding of Smad7 and Smad2/3 to the receptor site may be regulated stoichiometrically, which may account for the fact that, in cholesteatoma epithelium, Smad7 does not significantly inhibit Smad2 activation[17].

Apart from inhibiting Smad2 activation, Smad7 has been reported to play a critical role in mediating apoptosis by activation of the JNK signaling pathway[21]. However, we previously demonstrated that in cholesteatoma epithelium JNK signaling was not activated[22]. The decreased expression of Smad7 in cholesteatoma epithelium is in line with these findings and may be part of a protective signaling against apoptosis.

In this previous study we also demonstrated augmented ERK1/2/p38 MAPK signaling in cholesteatoma epithelium[22]. Moreover, it has been reported that in keratinocytes, TGFβ and MAPK signaling requires cooperative signaling to become more effective[23-25]. In a pilot experiment, we found significant correlations between TGFβ, pSmad2 and pERK1/2 in cholesteatoma (n=7; p=0.02 and p=0.03, unpublished results), but no correlations in control skin. These preliminary results indicate that, in cholesteatoma epithelium, TGFβ and pSmad2 may be involved in MAPK signaling. We hypothesize that, in cholesteatoma epithelium default levels of TGFβ may become effective because of cooperative signaling, leading to augmented transcription of several genes. One of these genes may be $p21^{cip1/waf1}$, which we previously found to be increased and associated with pERK1/2 expression[26].

This phenomenon as observed in cholesteatoma may also be of importance in the molecular understanding of transient *versus* irreversible epithelial mesenchymal transition (EMT). Cholesteatoma keratinocytes share many features with EMT, such as migration, spindle-like morphology, augmented ERK1/2 MAPK signaling and Akt activation[1,2,27,28] In particular cell types, irreversible EMT can be induced by Ras-Raf MAPK signaling alone[29], but in human keratinocytes both, TGFβ and MAPK signaling appear to be required[27]. In cholesteatoma, we have demonstrated a significantly increased ERK1/2 MAPK activation but a negligible activation of the TGFβ pathway. This finding, concomittant with the benign character of cholesteatoma, argues against irreversible EMT. This may indicate that the EMT characteristics in cholesteatoma are hallmarks of reversible EMT, which is a well known phenomenon in wound healing[30].

We therefore postulate that in cholesteatoma, the epithelial cells are subjected to a wound healing mechanism.

Cholesteatoma stroma

We found an increase in TGFβ and nuclear localization of pSmad2 in cholesteatoma stromal cells, but not a significant change in expression of the inhibitor Smad7. Stromal bio activation of TGFβ, however, is enhanced, which is clearly shown by the excessive deposition of fibronectin EDA. Recently, it has been demonstrated that in normal skin and in normal scarring fibroblasts, treatment with TGFβ increases Smad7 expression, whilst in keloid fibroblasts no TGFβ-mediated Smad 7 regulation was found[31]. In cholesteatoma stroma, although not increased, Smad7 expression was found to be correlated to TGFβ and pSmad2, indicating TGFβ controlled extracellular matrix deposition.

In healing wounds, FN is present at high levels and derived from two sources: plasma FN, which lacks the EDA segment and cellular FN, which contains EDA^+ FN and which is synthesized locally in the wound tissue[32]. At the wound site, the epithelium must migrate rapidly to cover the injury effectively to prevent deeper damages. In this process, FN EDA plays an important role because it provides a supporting scaffold that stimulates migration of epithelial and epidermal cells[32]. The FN-EDA presence in cholesteatoma stroma may therefore promote cell migration.

Evidence has been provided that TGFβ is important in wound healing and that one of its most important functions is the control of the inflammatory response[33]. The dominant role of TGFβ in the immune system is to induce tolerance as well as to maintain and resolve inflammation[34]. However, it appears that inflammation in cholesteatoma stroma is not down-regulated[1], but persistent for several reasons: 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[35]. 2) inflammation is recurrent because in most cholesteatoma, biofilms that can release endotoxins are present[36]. 3) inflammation persists because of lack of clearance in the enclosed cavity of the middle ear.

When inflammation does not cease, different cellular responses may occur. It has been reported that fibroblasts lose their ability to down regulate some of their ECM producing activities[34]. Moreover, with prolonged inflammation, increasing levels of TGFβ are produced in an attempt to control the inflammatory reaction[34]. Although in cholesteatoma stroma, numerous inflammatory cells are present which can inhibit TGFβ signaling, such as IFNγ and TNFα[1], it appears that in cholesteatoma stroma, this inhibition is subordinate to the signaling potency of TGFβ.

The conclusion of this study is that TGFβ bio-activation in cholesteatoma epithelium is not augmented, but still correlates to survival and migration. In the stroma, TGFβ bio-activation leads to excessive ECM deposition. These processes are symptoms of chronic wound healing, in which the balance between initiation and decrease of immune response is disturbed. We postulate that other aberrant cellular processes in cholesteatoma, such as increased angiogenesis and bone erosion can also be explained within the concept of cholesteatoma as a chronic wound healing process.

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Chapter 8

Summary and General Discussion

8

Summary and General Discussion

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

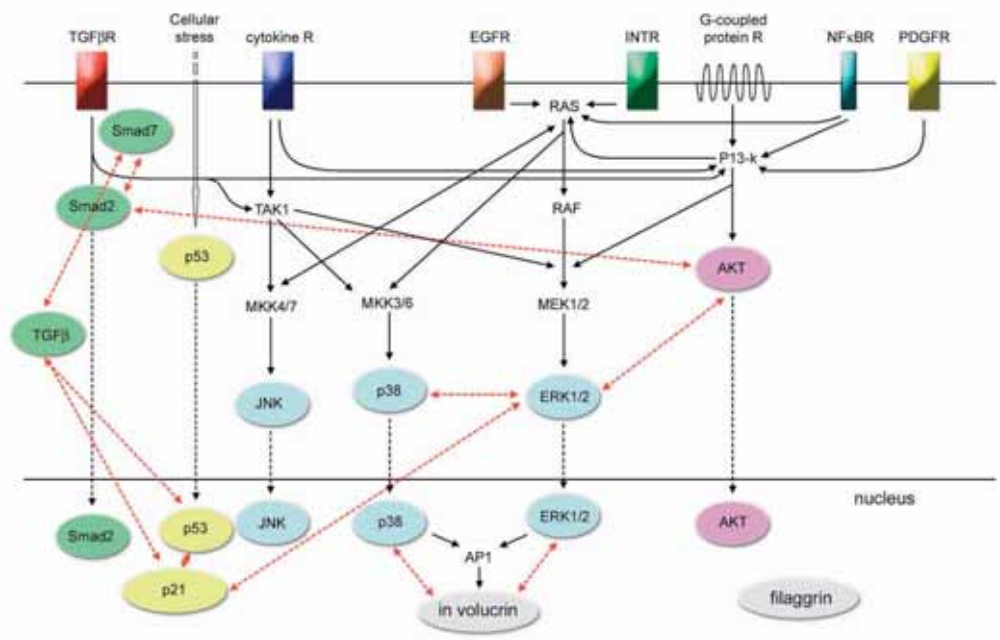

Figure 1. The pathways investigated in this thesis. The red lines represent significant correlations.

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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Samenvatting en Algemene Nabeschouwing

Samenvatting

In vergelijking met modern onderzoek zoals gen expressie profiling lijkt deze studie, die zich alleen richt op de expressie van eiwitten, tamelijk beperkt. Met betrekking tot het cholesteatoom zijn er echter sterke argumenten om de expressie van bepaalde eiwitten te onderzoeken. Cellulaire signalerings routes zijn activerings processen, die de cel motiveren tot een serie van reacties op een bepaalde toestand in of buiten de cel, bijvoorbeeld als er sprake is van cellulaire stress. Een dergelijke signalering is posttranscriptioneel, wat betekent dat activatie van een signaal niet het gevolg is van een toename van een bepaald eiwit, dus na transcriptie van het DNA, maar door het feit of een bepaald eiwit wel of niet geactiveerd is. Bovendien zijn in het cholesteatoom het epitheel en het stroma zodanig met elkaar verstrengeld (Hoofdstuk 7, fig. 1) dat het onmogelijk is om, bijvoorbeeld met eiwitsplitsende enzymen, het epitheel van het stroma te scheiden om met behulp van moleculaire technieken de verschillende cellen ervan te onderzoeken. Daarnaast, in de huid, hebben de cellen van de basale laag een ander eiwit expressie profiel dan de cellen in de suprabasale lagen en in de stratum corneum. Het onderzoek naar de aanwezigheid van verschillende eiwitten en de activatie daarvan in cellen in bepaalde huidlagen is daarom van belang in deze studie.

In **hoofdstuk 1** wordt het cholesteatoom beschreven vanuit een klinisch, morfologisch, biologisch en moleculair gezichtspunt. Verschillende theorieën met betrekking tot het ontstaan van het cholesteatoom worden besproken. Op grond van verschillende argumenten wordt onderscheid gemaakt tussen de benigne (goedaardige) kenmerken van het cholesteatoom en die van een maligniteit (kwaadaardige tumor). In dit hoofdstuk worden de kenmerken van wondgenezing en ontsteking in het cholesteatoom, en de hieruit volgende complexiteit van de eiwit signalering aangegeven.

In **hoofdstuk 2** wordt een gedetailleerde beschrijving gepresenteerd van de verschillende cellulaire signalerings routes en eiwitten, die onderzocht zijn in dit proefschrift. Deze routes, van celmembraan naar –kern, zijn de drie MAPKinases-, de pAkt- en de TGFβ signalerings routes. Bovendien wordt uiteengezet hoe p53 geactiveerd kan worden, wat de invloed van p53 is op apoptotische processen en wat de gecompliceerde rol is van $p21^{cip1/waf1}$ met betrekking tot de cel cyclus.

In **hoofdstuk 3**, zijn de verschillen in de mate van proliferatie, cel cyclus arrest en apoptose tussen cholesteatoom en controle huid vastgesteld. Onze resultaten laten zien, dat in het epitheel van het cholesteatoom Ki-67 verhoogd aanwezig is en dat dit vergezeld wordt van een toename van zowel p53 als van het cel cyclus arrest eiwit $p21^{cip1/waf1.}$ Wij toonden ook een significant positieve correlatie aan tussen p53 en $p21^{cip1/waf1}$. Er was minimaal apoptose in het epitheel van het cholesteatoom. Dit werd aangetoond door de actief Caspase 3 kleuring en de TUNEL test. Het is van buitengewoon belang in de immunohistochemie om de

juiste controles te gebruiken. Dit is maar al te waar gebleken met betrekking tot de TUNEL test, want de vals-positieve TUNEL kleuring was een belangrijk probleem dat in het begin van dit onderzoek opgelost moest worden. Het is veelbetekenend, dat in verschillende publicaties geen controles zijn gebruikt en/ of dat foto's van apoptotische cellen niet getoond worden, zodat deze studies niet betrouwbaar genoemd kunnen worden[1,2]. Het is van belang te onderkennen, dat zowel een apoptotische morfologie als een positieve apoptotische kleuring aanwezig moeten zijn om een cel apoptotisch te kunnen noemen, dit is helaas niet altijd het geval[3,4]. De resultaten van het onderzoek van het epitheel van het cholesteatoom geven aan, dat de verhoogde proliferatie niet gecompenseerd wordt door apoptose maar zou kunnen samengaan met cel cyclus arrest.

De resultaten van **hoofdstuk 4** laten zien, dat in cholesteatoom epitheel de Ras/ Raf/ ERK1/2 MAPKinase signalerings route betrokken is bij de p53-afhankelijke toename van $p21^{cip1/waf1}$ expressie. Dit werd bevestigd door de gecorreleerde expressie van p53 en $p21^{cip1/waf1}$ en die van $p21^{cip1/waf1}$ en geactiveerde ERK1/2. Interessant is dat activatie van ERK1/2 geassocieerd wordt met zowel stimulatie als met remming van cel proliferatie. De sterkte en de duur van de ERK1/2 activatie bepaalt het antwoord van de cel: proliferatie of cel cyclus arrest. De door ERK1/2 bewerkstelligde opregulatie van proliferatie vereist een tijdelijk, laag ERK1/2 activeringssignaal, terwijl opregulatie van $p21^{cip1/waf1}$ een sterk en langdurig ERK1/2 activeringssignaal vereist. In het epitheel van het cholesteatoom zijn deze verschillende processen zichtbaar door de enkelvoudige ERK1/2 expressie in het proliferatieve compartiment. In de suprabasale lagen, waar langdurige ERK1/2 expressie aanwezig is, zijn de cellen ook positief bevonden voor het cel cyclus arrest eiwit $p21^{cip1/waf1}$. Aanhoudende remming van de cel cyclus veroorzaakt stapeling van epitheliale cellen die in G^0-arrest zijn[5]. Wij concluderen daarom, dat in het cholesteatoom overheersende cel cyclus arrest zou kunnen bijdragen aan epitheliale hyperplasie.
Veranderingen in specifieke signaleringsroutes zouden de abnormale terminale differentiatie kunnen verklaren van de keratinocyten in het cholesteatoom.

In **hoofdstuk 5** is de correlatie onderzocht tussen terminale differentiatie en signalering via de MAPKs. In dit hoofdstuk is aangetoond dat de aanwezigheid van geactiveerd ERK1/2 en –p38 positief geassocieerd is met de expressie van involucrine. Geactiveerd JNK lijkt niet betrokken te zijn bij dit proces. Dit geeft aan, dat terminale differentiatie in het epitheel van het cholesteatoom geschiedt via activatie van ERK1/2 en p38 signaleringsroutes. Onze resultaten zijn in tegenspraak met eerdere publicaties waarin p38 signalering het belangrijkste reguleerend mechanisme wordt genoemd[6]. Wij konden ook niet de bevinding van Efimova bevestigen met betrekking tot de p38-gerelateerde reductie van ERK1/2 activiteit[7]. In dit hoofdstuk wordt beargumenteerd dat groeifactor receptor activatie en andere pro-inflammatoire responsen een parallelle ERK1/2 and p38 signalering teweeg zou kunnen brengen. Een andere reden voor autonome ERK1/2 activatie zou kunnen zijn dat keratinocyten in het cholesteatoom onderworpen zijn aan een

overlevingsprogramma veroorzaakt door verminderde cel-matrix hechting. Het is aangetoond, dat de basaal membraan van het cholesteatoom afwijkend en onderbroken is[8]. Bescherming van cellen tegen apoptose door verminderde cel-matrix binding is geassocieerd met, en vereist een langdurige ERK1/2 MAPK activatie[9]. De aanname dat cholesteatoom keratinocyten onderworpen zijn aan een dergelijke hechtings-onafhankelijke overleving is in overeenstemming met onze eerder aangetoonde langdurige ERK1/2 activatie en minimale apoptose[10,11]. Het opmerkelijke is dat dit overlevingsmechanisme van belang is bij de voortgang van weefselherstel en, in de huid, voor de migrerende keratinocyten aan de frontlinie van de wond. De argumenten van een parallele signalering ten gevolge van een door inflammatie geinduceerde stress respons of van een keratinocyten-overlevingsprogramma gedurende wondgenezing kunnen met betrekking tot het cholesteatoomweefsel zeker waar zijn (persoonlijke communicatie met Efimova).

De bevinding in **hoofdstuk 6** was dat het overlevingseiwit pAkt/PKB significant verhoogd is ten opzichte van controle huid. Deze waarneming valt samen met de resultaten uit eigen eerdere publikaties waarin minimale apoptose is aangetoond in het epitheel van het cholesteatoom. Het eiwit dat betrokken is bij late terminale differentiatie, filaggrine, werd significant verlaagd aangetroffen en hoewel aangetoond is dat PI3K/Akt signalering betrokken is bij terminale differentiatie, konden wij geen associatie aantonen tussen pAkt en filaggrine[12]. Involucrine, dat eveneens betrokken is bij terminale differentiatie, was significant verhoogd, maar ook tussen pAkt en involucrine konden we niet een relatie aantonen. In psoriasis echter, is melding gemaakt van eenzelfde differentiatie profiel: een verhoogde involucrine expressie en een afwezigheid of verlaging van de hoeveelheid filaggrine[13,14]. Er is gesuggereerd dat abnormale structuren van adhesiemoleculen verantwoordelijk zouden kunnen zijn voor de ontregeling in productie van de componenten van de cornified envelope[15]. Deze suggestie is onlangs ondersteund door een artikel van Calautti, waarin bewezen werd dat de cadherine-catenine adhesie complexen nodig zijn om tot een differentiatie-specifieke activatie van PI3K te komen[12]. Kortom, bescherming tegen apoptose geschiedt door middel van geactiveerde Akt, maar voor de aanzet tot late differentiatie is nog een andere component nodig: cel adhesie. Bij de analyse van de integriteit van cholesteatoom weefsel vonden Naim et al. dat, in tegenstelling tot normale huid, beta-catenine verminderd dan wel afwezig was in de suprabasale lagen van het cholesteatoom[16]. Dit impliceert dat, in overeenstemming met onze eerdere publicaties, verminderd of afwezig cellulair contact in cholesteatoom epitheel de oorzaak kan zijn van zowel verhoogde involucrine als van een verlaagde filaggrine expressie.

Van TGFβ wordt aangenomen dat het een spilfunctie inneemt bij wond genezing en de onderzoeksvraag van **hoofdstuk 7** was of in het cholesteatoom, de TGFβ signalerings route is geactiveerd.

In de epithelia van cholesteatoom en huid vonden we overeenkomstige expressies van TGFβ en geactiveerd Smad2 (pSmad2), maar in het cholesteatoom

was de expressie van de antagonist Smad 7 verlaagd ten opzichte van normale huid. De correlaties tussen TGFβ, pSmad2 en Smad7 zijn een indicatie dat Smad activatie en remming beide operationeel zijn. Desalnietemin, ondanks de verlaagde Smad7 expressie leiden deze processen niet tot een significante toename van pSmad2. Het is vermeld dat behalve de remming van Smad2 activatie, Smad7 een belangrijke rol lijkt te spelen bij het bewerkstelligen van apoptose door activatie van de JNK signalerings route. De verlaagde Smad7- en onze eerder aangetoonde niet verhoogde JNK expressie in cholesteatoom epitheel zou daarom kunnen duiden op bescherming tegen apoptose. In dit hoofdstuk vergelijken we in een pilot studie de resultaten van deze studie met die van de resultaten van ons onderzoek naar de MAPKinase signalerings route en we hebben correlaties gevonden tussen TGFβ, pSmad2 en pERK1/2 ($p=0.02$ and 0.03). Wij veronderstellen dat in het epitheel van het cholesteatoom, een standaard niveau van TGFβ effectief kan worden door samenwerking met andere signalerings routes, en dat dit weer kan leiden tot verhoogde transcriptie van verschillende genen. Een van deze genen zou $p21^{cip1/waf1}$ kunnen zijn, dat we eerder al verhoogd aanwezig hebben gevonden en dat bovendien gerelateerd is aan ERK1/2 expressie[10]. Dit kan een fijn regulerings mechanisme zijn van de TGFβ signalering, wat aangeeft dat eiwit signalering verhoogd kan zijn, maar sterk gecontroleerd.

In het stroma van het cholesteatoom zijn TGFβ and pSmad2 verhoogd, terwijl de Smad7 expressie gelijk was aan die in controle huid. EDA-fibronectine (EDA-FN) accumulatie in het stroma van het cholesteatoom was excessief, terwijl er geen EDA-FN aantoonbaar was in controle huid. In dit hoofdstuk wordt beargumenteerd dat de verhoogde stromale expressies van TGFβ, pSmad2 en EDA-FN, het cellulaire antwoord is op een langdurige ontsteking. Er zijn verschillende redenen voor een dergelijke hypothese:1) het cholesteatoom kan worden beschouwd als een "vreemd lichaam", dat in het middenoor een steeds terugkerende (=chronische) ontstekingsreactie kan veroorzaken[17]. 2) De inflammatie is chronisch omdat in de meeste cholesteatomen biofilms aanwezig zijn die endotoxinen en bacteriën kunnen vrijmaken[18]. Evenals bacteriën kunnen endotoxinen opnieuw de ontsteking initiëren door de cellen aan te zetten tot de productie van cytokinen. 3) inflammatie kan zichzelf consolideren door het gebrek aan clearance in de omsloten ruimte van het midden oor. Er kan ook een combinatie mogelijk zijn van deze drie factoren, of ze zouden allemaal een rol kunnen spelen in de chroniciteit van het cholesteatoom. In dit hoofdstuk geven we aan, dat het cholesteatoom een proces is van chronische wondgenezing, waaraan de TGFβ signalerings route en de voortdurende ontsteking lijken bij te dragen

Algemene Nabeschouwing

In het epitheel van het cholesteatoom zijn verhoogde expressies gevonden van eiwitten die betrokken zijn bij proliferatie (Ki-67), cel cyclus arrest (p53 en $p21^{cip1/waf1}$), voortijdige terminale differentiatie (involucrine) en overleving (pAkt). Bovendien is aangetoond dat de activatie van MAPK- verhoogd is en geassocieerd is met de TGFβ signalerings route. De eiwitten waarvan de expressie verlaagd was, hebben betrekking op late terminale differentiatie (filaggrine) en apoptose (actief Caspase3). We hebben verschillende correlaties gevonden tussen de verschillende eiwitten die onderzocht zijn. We veronderstellen, dat deze correlaties binnen de context passen van samenhangende signalerings processen. Het lijkt dat deze connecties in zowel de MAPK- als de TGFβ signalering, cel cyclus arrest, vroege terminale differentiatie en overleving ondersteunen, wat kenmerkend is voor wondgenezing. Samenvattend suggereert dit, dat het epitheel van het cholesteatoom zich gedraagt als een wond genezingsproces met migrerende keratinocyten. Veel biologische karakteristieken van het epitheel van het cholesteatoom kunnen verklaard worden met behulp van dit concept. Verhoogde proliferatie, aangetoond door de proportioneel verhoogde Ki-67, wordt niet gecompenseerd door verhoogde apoptose. Dit kan hyperplasie verklaren, wat een normaal fenomeen is in het cholesteatoom. Hypertrofie is ook frequent aanwezig en kan veroorzaakt worden door cel cyclus arrest, maar ook door differentiatie, omdat deze twee processen het cellulair volume doen toenemen[19,20]. Bovendien is het aangetoond dat keratinocyten in een wondgenezingsproces hypertroof zijn (wat derhalve een gevolg is van cel cyclus arrest en afwijkende differentiatie)[21]. Afwijkende differentiatie, door de voortijdige expressie van involucrine maar ook door de vermindering van filaggrine, kan veroorzaakt worden door verlies van adhesie[12,15]. Dit verlies van adhesie kan veroorzaakt zijn door een onderbroken en veranderde basaal membraan en/of een verminderde α catenine expressie. Beide verschijnselen zijn eerder aangetoond in het epitheel van het cholesteatoom[8,16]. Bovendien is het gebrek aan adhesie zichtbaar omdat in verschillende publikaties op foto's van een cholesteatoom een verwijding van de intercellulaire ruimtes te zien is, dit laatste is ook vermeld als een fenomeen in wond genezing[21]. De afname van filaggrine kan ook een ander biologisch kenmerk van het epitheel van het cholesteatoom verklaren: dat van parakeratose. Als de late terminale differentiatie niet opgestart wordt, behouden de cellen hun kernen en wordt het weefsel parakeratotisch. Interessant is dat vroege terminale differentiatie, zoals aangetoond door involucrine expressie, de proliferatie niet doet stoppen, want involucrine-positieve cellen zijn nog in staat om DNA te synthethiseren[19]. De toename van TGFβ en pSmad2 in het stroma van cholesteatoom en de verhoogde EDA-fibronectine duiden ook op een chronisch wondgenezingsproces. In het cholesteatoom is dit proces duidelijk zichtbaar door de accumulatie van EDA-fibronectine. Hoewel chronisch, nemen we aan, dat dit toch een onderdeel is van normale wondgenezing want EDA-fibronectine is noodzakelijk voor normale, zelfbeperkende wondgenezing[22]. Bovendien, bij normale wondgenezing is het laatste signaal dat van de verdwijning van de ontstekingscellen. Het is vermeld dat dit gebeurt door middel van apoptose[23]. In hoofdstuk 3, hoewel er minimaal apoptose

was in het epitheel, vonden we vele apoptotische cellen in het stroma. De recurrency van het cholesteatoom kan ook worden verklaard, want cellen kunnen, zelfs vele jaren na verwonding, opnieuw geactiveerd worden[24].

Samenvattend, in het cholesteatoom lijkt cellulaire signalering te verlopen langs de lijnen van normale, maar chronische wondgenezing.

Toekomstig onderzoek.
Wij adviseren onderzoek te verrichten naar farmacologische interventies gericht op ontstekings controle. Omdat de toediening van antibiotica niet erg succesvol is gebleken- waarschijnlijk wegens de aanwezigheid van biofilms- denken wij dat het gebruik van antimicrobiële peptiden een geschikte nieuwe therapie zou kunnen zijn.
Om meer inzicht te verwerven in het ontstaan van het cholesteatoom stellen wij voor om de invloed van externe stimuli, zoals keratine deeltjes en endotoxinen, op de eiwitexpressie en het eiwitsignaal profiel te bestuderen in het advancing front van huid uit de buitenste gehoorgang en midden oorslijmvlies[25]. Retractiepockets worden beschouwd als een voorstadium van cholesteatoom vorming, hoewel de daadwerkelijke ontwikkeling tot een cholesteatoom niet te voorspellen is. Het is daarom van belang om de eiwitexpressie en het eiwit signaleringsprofiel van retractiepocket weefsel te vergelijken met dat van cholesteatoom. Wij doen daarnaast nog de aanbeveling om te bestuderen in hoeverre biofilms een rol spelen in de ontwikkeling en voortgang van het cholesteatoom. Dit zou gedaan kunnen worden door middel van de detectie van zowel bacteriekolonies als van hun glycocalix laag in retractiepockets en in verschillende soorten cholesteatoom.

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CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 9 juni 1953 te Deventer. In 1969 behaalde zij haar MULO-diploma aan de Kon. Emmaschool te Deventer, waarna zij van 1969 tot 1971 HBO-A chemie studeerde aan de Deventer Laboratoriumschool. De HBO studie werd als HBO-B voortgezet aan het Van Leeuwenhoek instituut te Delft alwaar de biochemische studierichting werd gevolgd. Tegelijkertijd werd aan de Hogeschool Delft een cursus radio-isotopen met goed gevolg voltooid. Het HBO-B diploma werd in 1972 behaald, waarna in datzelfde jaar een betrekking als analist bij de afdeling pathologie aan de Leidse Universiteit werd aanvaard. Van 1976 tot en met 1999 heeft zij als part-time research analiste gewerkt bij het laboratorium prenatale diagnostiek van de afdeling verloskunde van het Academisch Ziekenhuis te Leiden. Dit heeft geresulteerd in verschillende artikelen in vakbladen. In 2000 heeft zij een eveneens part-time betrekking als research analist aanvaard bij de afdeling KNO. Bij deze afdeling heeft ze vanaf 2002 als promovenda een volledige aanstelling. Sinds September 2005 is zij wetenschappelijk medewerker van het oto-biologisch laboratorium van de afdeling KNO, waarvan het onderzoek zich met name richt op de binnenoorpathologie en -regeneratie.

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Abbreviations

Akt	AKT8 virus oncogene cellular homolog
Apaf-1	Apoptotic protease activating factor
APO1	apoptosis-related protein
ARF	Alternative reading frame
ASK1	Apoptosis signal-regulating kinase 1
AP1	Activator protein 1
BAD	Bcl2-antagonist of cell death
Bcl-2	B-cell lymphomal leukaemia-2
Bcl-XL	B-cell lymphomal leukaemia-XL
BMP	Bone morphogenetic proteins
CAD	Caspase-activated DNAse
CAK	Cyclin activating kinase
cAMP	Cyclic adenosin monophosphate
Caspase	Cysteine proteases with aspartate specifity
CBP	CREB binding protein
CDK	Cyclin-dependent kinase
CHK	Checkpoint kinase
CIP1	Cdk-interacting protein
CREB	cAMP response element-binding protein
Crk	CT10 sarcoma oncogene cellular homolog
DAB	Diaminebenzidinetetrahydrochloride
DNA	Desoxyribo nucleic acid
DR	Death receptor
E2F	early-region-2 transcription-factor-
ECM	Extra cellular matrix
EGF	Epidermal growth factor
Erk	Extracellular signal-regulated kinase
FADD	Fas-associated protein with death domain
FAK	Focal adhesion kinase
FEN1	Flap endonuclease 1
FH	Forkhead
GADD45	Growth arrest and DNA damage protein 45
GSK3	Glycogen synthase 3
GRB2	Growth factor receptor-bound protein
GTP	Guanine nucleotide tri phosphate
IGF	Insulin-like growth factor
IkB	Inhibitor of NFκB
IKK	IkappaB kinase
ILK	Integrin-linked kinase
JNK	c-Jun N-terminal kinase
KILLER	Killer Cell Immunoglobulin-Like Receptor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase

MAPKAP	MAP kinase activated protein kinase
MDM2	Murine double minute 2
MEK	MAPK/Erk kinase
MEKK	MAPK/Erk kinase kinase
NF-κB	Nuclear factor kappa B
NIK	NF-κB inducing kinase
PCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PdGF	Platelet-derived growth factor
PDK1	Phosphoinositide dependent kanse-1
PI3K	Phosphoinositide 3 kinase
PIP2	Phosphatidylinositol 3,4-biphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PTEN	Phosphatase and tensin homologue
PUMA	p53 upregulated modulator of apoptosis
RasGRP	Ras guanyl nucleotide-releasing protein
Rb	Retinoblastoma protein
Rel	Reticuloendotheliosis oncogene cellular homolog
SAPK	Stress-activated protein kinase
SARA	Smad anchor for receptor activation
Shc	SH2-containing collagen-related proteins
Smad	contraction of Sma and Mad (Mothers against decapentaplegic)
Smurf	Smad ubiquitination regulatory factor
SOCS	Suppressor of cytokine signaling
SOS	Son of sevenless guanine nucleotide exchange factor
SOX	Sry-box transcription factors
Src	Rous sarcoma oncogene cellular homolog
TAK1	Transforming growth factor-β (TGF-β)-activated kinase 1
TAB	TAK-binding protein
TBS	Tris-buffered saline
TGFβ	Transforming growth factor beta
Thr	Threonine
TNFα	Tumor necrosis factor alpha
TPA	12-o-tetradecanoylophorbol-13-acetate
TRADD	TNF receptor-1-associated death domain protein
TRAF	TNF-receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
WAF1	Wild-type p53-activated fragment