Cellular signaling in human cholesteatoma

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Chapter 7

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

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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

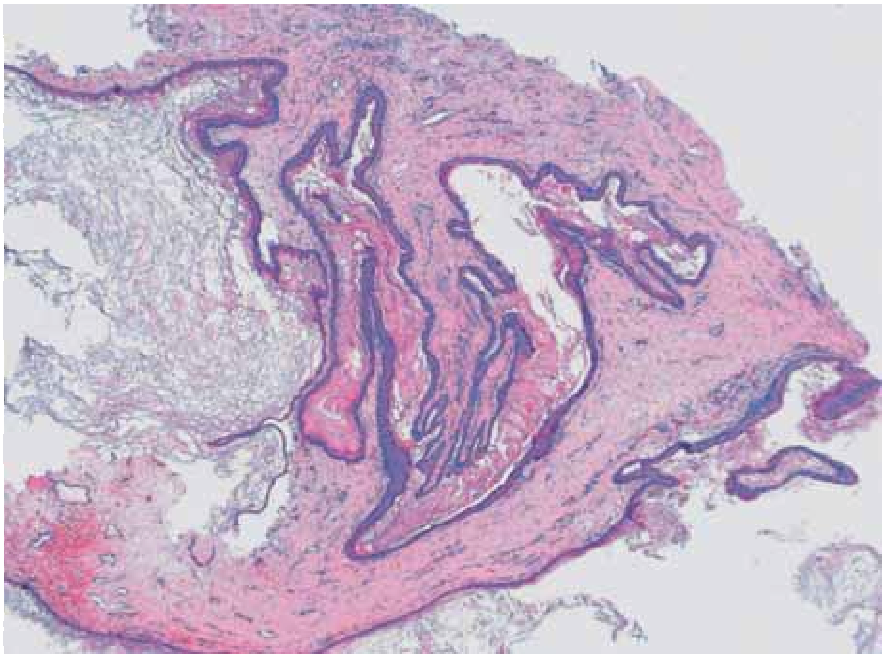

Figure 1. Hematoxylin-eosin staining of cholesteatoma. Clearly visible is the entanglement between epithelium and connective tissue.

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

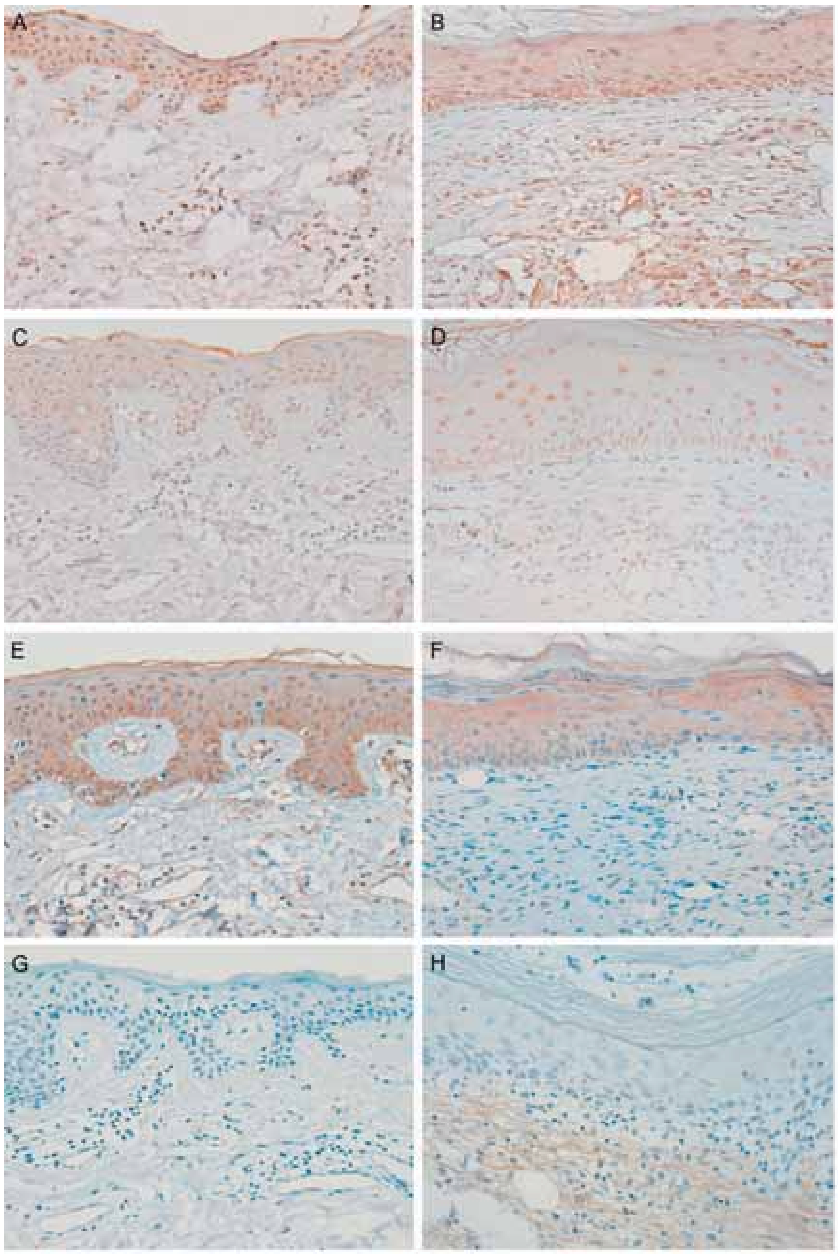

Figure 2. Immunohistochemical localization of TGFβ, pSmad2, Smad7 and fibronectin EDA in paraffin sections of control retro-auricular skin (A, C, E, G) and human cholesteatoma (B, D, F, H). TGFβ-positive cells are prominently expressed in both, epithelium and stroma (A,B). pSmad2-positive cells scatter 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

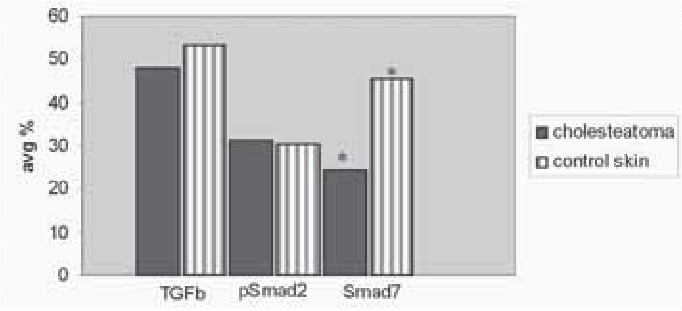

Figure 3. Represents average percentages of TGFβ, pSmad2 and Smad7 in cholesteatoma epithelium and control skin.

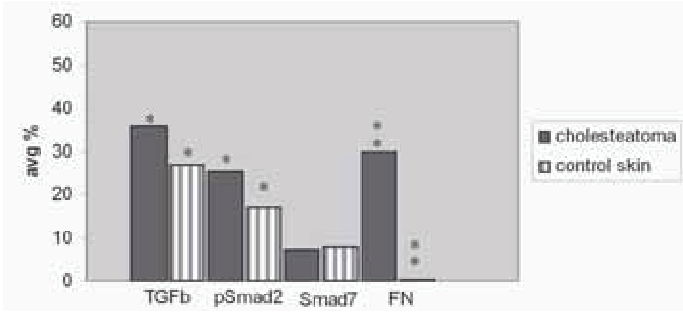

Figure 4. Represents average percentages of TGFβ, pSmad2, Smad7 and fibronectin EDA in cholesteatoma stroma and the stroma of control skin.
*= p<0.05; **= p<0.001.

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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