REVIEW

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Molecular biology of cholesteatoma

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Abstract

Cholesteatoma is a non-neoplastic, keratinizing lesion, characterized by the proliferation of epithelium with aberrant micro-architecture into the middle ear and mastoid cavity. The exact pathogenic molecular mechanisms behind the formation and propagation of cholesteatoma remain unclear. Immunohistochemical examinations of the matrix and perimatrix have considerably improved the knowledge of cholesteatoma pathogenesis. In this review, the current concepts of cholesteatoma pathogenesis are discussed. Currently, the most widely acknowledged pathogenesis of acquired cholesteatoma is the theory that negative pressure, dysfunction of the Eustachian tube, causes a deepening retraction pocket that, when obstructed, desquamated keratin cannot be cleared from the recess, and a cholesteatoma results. Local infection leads to a disturbance of self-cleaning mechanisms, with cell debris and keratinocytes accumulate inside the retraction pocket, and this is followed by an immigration of immune cells, *i.e.*, Langerhans' cells, T-cells, macrophages. There is an imbalance and a vicious circle of epithelial proliferation, keratinocyte differentiation and maturation, prolonged apoptosis, and disturbance of self-cleaning mechanisms. The inflammatory stimulus will induce an epithelial proliferation along with expression of lytic enzymes and cytokines. Bacteria inside the retraction pocket produce some antigens, which will activate different cytokines and lytic enzymes. These cytokines lead to activation and maturing of osteoclasts with the consequence of degradation of extracellular bone matrix and hyperproliferation, bone erosion and finally progression of the disease. Further research is necessary for a better understanding of the pathogenetic mechanisms and to expand the spectrum of therapeutic options.

Keywords: cholesteatoma, molecular biology, inflammatory mediators, proliferation markers, gene expressions.

Introduction

Cholesteatoma is a progressive, benign epithelial lesion, which destroys the bony structures of the middle and sometimes the inner ear, characterized by an expanding growth consisting of keratinizing squamous epithelium in the middle ear and/or mastoid [1]. It is considered more aggressive during childhood. The exact pathogenetic molecular mechanisms behind the formation and propagation of cholesteatoma are still unclear. This review is focused on understanding the cellular mechanisms leading to the development, progression, and bone destruction in cholesteatomas. A better knowledge of the mechanisms involved could lead to new strategies for the non-surgical prevention and management of this disease.

Middle ear cholesteatomas are characterized by intense cell proliferation accompanied by the consequent accumulation of keratin debris and the destruction of bone structures surrounding the temporal bone [2]. Bone lysis and recurrence are relevant features in the pathophysiology of cholesteatoma, making it a dangerous, debilitating and difficult to treat condition.

The common presenting symptoms are otalgia, malodorous otorrhea, and hearing loss. Its potential for causing complications in the central nervous system (*e.g.*, brain abscess, meningitis) makes it a potentially fatal lesion. These complications that may occur are usually secondary to chronic poly-microbial infection and bony erosion. The only effective treatment for cholesteatoma is surgery. To date, there are no medical treatments that have been proven effective for this disease, except the treatment of the secondary infectious processes. The pathogenesis of middle ear cholesteatoma is still unknown and subject of controversial discussions.

Cholesteatomas may be either congenital or acquired.

Congenital cholesteatoma

Congenital cholesteatoma, by definition originate from areas of keratinizing epithelium within the middle ear cleft. Several theories emerged including: (a) the presence of an ectopic epidermis rest, (b) in-growth of meatal epidermis, (c) metaplasia following infection/inflammation, and interestingly, (d) reflux of amniotic fluid containing squamous epithelium *in utero* into the middle ear (Figure 1). However, the epithelial rest theory is most commonly accepted. Derlacki and Clemis [3] defined congenital cholesteatomas as "epithelial inclusions behind an intact TM in a patient without a history of otitis media". Michaels [4] in 1980s identified squamous cell tuft present in the temporal bones of fetuses aged 10–35 weeks. This "epidermoid formation" was present in the anterior and superior region of the middle ear cleft. Failure of involution could be basis of congenital cholesteatoma.

Karmody *et al.*, in 1998 [5], presented the histological documentation of congenital cholesteatoma with a squamous epithelial rest in neonatal temporal bones. Due to their tendency to grow medially towards the sinus tympani [6] and due to a high submucosal element [7] congenital cholesteatomas have a high rate of recurrence after removal [8].

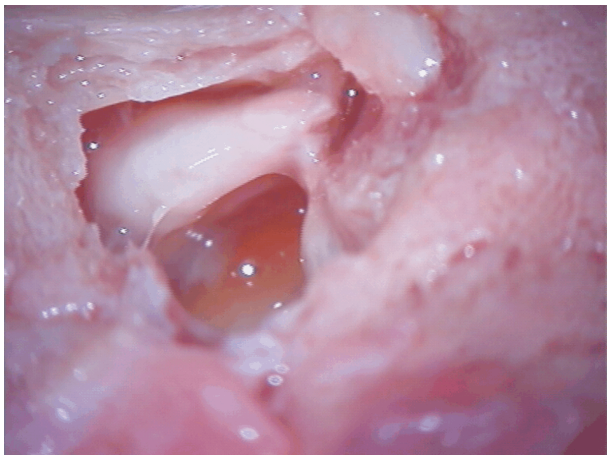

Figure 1 – ***New born temporal bone. Right ear. Superior view of the tympanic cavity filled with amniotic fluid.***

Acquired cholesteatoma

Although the pathophysiology of the acquired cholesteatoma remains to be clearly elucidated, it is presumed to be multifactorial, as many theories have been proposed and investigated. Iatrogenic or non-iatrogenic tympanic membrane trauma like perforation, displacement, retraction or invagination, tympanic membrane disease, tympanic cavity mucosa disease, ear infection, and Eustachian tube dysfunction are likely to trigger acquired cholesteatoma development [9]. There are four basic theories of acquired aural cholesteatoma pathogenesis: (1) invagination of the tympanic membrane (retraction pocket cholesteatoma), (2) basal cell hyperplasia, (3) epithelial in-growth through perforation (the migration theory), and (4) squamous metaplasia of middle ear epithelium. Sudhoff and Tos [10] proposed a combination of invagination and basal cell theories as an explanation for the retraction pocket cholesteatoma formation. The most widely acknowledged pathogenesis of acquired cholesteatoma is the theory that negative pressure causes a deepening retraction pocket that, when obstructed, desquamated keratin cannot be cleared from the recess, and cholesteatoma results. The origin of such retraction pocket cholesteatomas is thought to be the dysfunction of the Eustachian tube or otitis media with effusion with resultant negative middle ear pressure (*ex vacuo* theory). Usually, the pars flaccida, being less fibrous and less resistant to displacement, is the source of the cholesteatoma [11]. Chronic inflammation seems to play a fundamental role in multiple etiopathogenic mechanisms of acquired cholesteatoma.

The histological description of cholesteatomas was made in 1972 by Lim and Saunders [12]. Cholesteatoma has a keratinized stratified squamous epithelium with four layers identical to normal epidermis, Langerhans cells, and keratin-hyaline granules. This epithelium was named cholesteatoma matrix. The perimatrix was observed in the periphery of the matrix consisting of connective tissue, containing collagen fibers, fibrocytes and inflammatory cells. Immunohistochemical examinations of the matrix and perimatrix have considerably improved the knowledge about the cellular structure of cholesteatoma.

Florid inflammation and angiogenesis are distinctive features of the perimatrix in most cases (Figure 2).

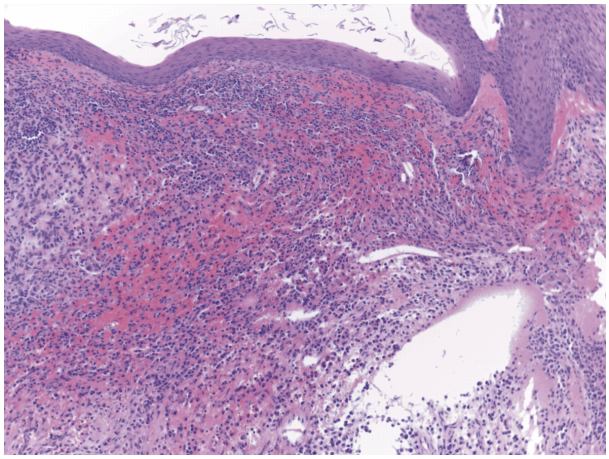

Figure 2 – ***Hematoxylin–Eosin staining (×100) of acquired cholesteatoma specimen. Pathologic specimen shows a thick keratinized epithelial edge surrounding a highly vascularized and inflammatory matrix, with a plethora of mast cells, polymorphonuclears and granulation tissue.***

Biology of cholesteatoma

Studies published so far presented many data about the biology of cholesteatomas, but many questions remain unanswered. Although cholesteatoma is hyperproliferative, it does not exhibit typical features of neoplasia. It does not metastasize, nor is it genetically unstable. The induction of cholesteatoma formation seems to be related to both internal molecular dysregulation and external stimuli in the form of pro-inflammatory cytokines, growth factors and/or bacterial toxins. There is an imbalance and a vicious circle of epithelial proliferation, keratinocyte differentiation and maturation, prolonged apoptosis, and disturbance of self-cleaning mechanisms. Bacteria inside the retraction pocket produce some antigens, which will activate different cytokines and lytic enzymes, these cytokines lead to the activation and maturation of osteoclasts with the consequence of degradation of extracellular bone matrix and hyperproliferation, bone erosion and finally progression of the disease [13].

The role of inflammatory mediators in the pathogenesis of cholesteatoma

The immune system responds to injury or irritation through an innate cascade known as inflammation. The main actors in this process are the inflammatory mediators, which include proteins, peptides, glycoproteins, cytokines, arachidonic acid metabolites (prostaglandins and leukotrienes), nitric oxide, and oxygen free radicals. These compounds are produced by epithelial cells, endothelial cells and infiltrating inflammatory cells. Inflammatory mediators are a double-edged sword, having the potential to fight off infection, but also to damage the host [14].

Acquired cholesteatomas almost universally arise in the setting of inflammation and infection. Endotoxin, a component of bacterial wall is considered responsible for the initiation of inflammation in the middle ear. It stimulates local macrophages to produce tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β). Keratinocytes respond to injury by producing many soluble mediators, independently from the immune cells, including: TNF-α, IL-1β, IL-6, and IL-8 [15]. The consequence is the formation of inflammatory granulation tissue with invading epithelium in active human cholesteatomas as well as in experimental animal cholesteatomas. As in wound healing and other pathologic conditions, cholesteatomas consist of activated keratinocytes, which have become migratory and proliferative.

The pathobiologic reason for the observed activation of proliferation in cholesteatoma tissue is not completely understood. It is theorized that the intense infiltration of cholesteatoma by immune cells leads to chronic inflammation *via* continuous overproduction of certain cytokines. This continuous inflammatory state is probably the result of the presence of granulation tissue, bacterial pathogens and hyperproliferating keratinocytes [13]. Studies have shown that a large number of mast cells can be found in acquired cholesteatoma [16] as well as increased number of activated T-cells and macrophages [17].

Cytokines are proteins that are produced by cells as a response to inflammatory processes. They act on cellular intercommunication and regulate cell division, thereby acting as growth factors [18]. The inflammatory process that occurs in the cholesteatoma perimatrix seems to have an important role in inducing the production of cytokines by epithelial cells.

Among the involved cytokines, TNF-α plays a major role in the pathogenesis of cholesteatoma, being found increased mainly in the connective tissue and epithelium of cholesteatoma samples, compared to the normal canal skin. The levels of TNF-α were correlated with the amount of inflammatory cells infiltration, bony destruction and severity of infection [19]. TNF-α is secreted by activated macrophages, mast cells and also keratinocytes. The effect of increased production of TNF-α is an osteoclast mediated bone resorption [20], stimulation of collagenase and prostaglandin E secretion by fibroblasts, which will lead to soft tissue destruction [21] and can also cause the resorption and inhibition of proteoglycans in the cartilage [22].

Also, IL-1 (both IL-1α and IL-1β) was found increased in the epidermis of cholesteatoma compared to the normal squamous epithelium [23, 24] playing an important role in the bony resorption process and also stimulating the proliferation of keratinocytes [25]. The stimulation of cultured cholesteatoma epithelial cells by lipopolysaccharide (LPS) led to an increased synthesis of IL-6 and IL-8, while Dexamethasone had a significant inhibitory effect [26] suggesting the importance of inflammation in the pathogenesis of cholesteatoma.

Enzymatic activity in cholesteatomas

Recent studies showed that variations in cellular production of matrix metalloproteinases (MMPs) and their specific inhibitors (TIMPs) contribute to the pathophysiology of cholesteatoma, especially in the development of bone erosion. They are involved in the physiologic turnover of the cholesteatoma epithelium matrix [2].

MMPs are zinc and calcium-dependent endopeptidases synthesized by different types of cells such as fibroblasts, keratinocytes, macrophages, and endothelial cells activated by proteolysis [27]. MMPs proteolytic activity is controlled by their precursors during activation and inhibited by endogenous inhibitors, alpha macroglobulins, and TIMPs [2, 27].

Normally, their activity is tightly controlled, as an increase in their activation would cause denudement of the extracellular matrix and increased invasiveness by the epithelium. In cholesteatoma, studies have indicated a clear imbalance in the regulation of MMPs, with an overall up-regulation of MMP expression and a decrease in MMP inhibitors resulting in degradation of the extracellular matrix. Specific cholesteatoma MMP isoenzymes (MMP2, MMP3, and MMP9) were first identified in 1996 [27] with the use of immunohistochemistry tests. Since then, immunohistochemistry has been extensively used to analyze and compare changes in enzyme levels in cholesteatomas and healthy tissues. Increased levels of MMP9, MMP2 and MMP1 have been reported. Immunolabeling of MMP1, 2, 3 and 9 was observed mainly in the basal and suprabasal layers of the cholesteatoma epithelium; MMP9 was specifically seen in areas with inflammatory cell infiltration [28]. The tissue inhibitor of metalloproteinases (TIMP-1) could be detected only in very limited areas of the granulation tissue could be detected only in very limited areas of the granulation tissue [29]. Collagenase is also involved in the local invasion process by aural cholesteatoma, stimulating the osteoclastic resorption by degrading the osteoid surface of the bone and thus facilitating osteoclastic activity [30].

This enzimatic activity causes the aggressiveness seen in cholesteatoma concerning its invasiveness and bony destruction [27]. According to Visse and Nagase [28], MMP2 levels were increased after exposure to LPS or TNF-α in cultured gerbil TMs. This supports a link between inflammatory mediators and the secretion of potentially destructive MMPs in the TM, which may contribute to the pathogenesis of cholesteatoma. Gene expression of matrix metalloproteinases and their inhibitors has been verified in cholesteatomas by Real-Time PCR. The results showed the presence of mRNA in MMP1, MMP2, MMP3 and TIMP-1 but not MMP9. There were not found correlations of MMP and TIMP expressions with the aggressiveness of the disease [31].

Growth factors in cholesteatomas

However, the presence of cytokines alone is insufficient to explain the aggressive behavior of cholesteatoma, which also depends on the interaction between these proteins and their binding receptors on the epithelium of the cholesteatomas [32]. The higher growth rate of cholesteatoma cultures may be explained by a higher growth factor activity.

Epidermal growth factors (EGF) stimulate proliferation and differentiation of epidermal cells, fibroblasts and endothelial cells. Transforming growth factor alpha (TGF-α)

is also an important growth mediator for epithelial and mesenchymal cells. Several immunohistochemical studies have reported abnormal growth factor ligand and receptor expression in cholesteatoma biopsies, caused by defective regulation of epidermal growth factor receptor and increased interferon (IFN)-γ receptor, platelet derived growth factor (PDGF), TGF-α, IL-1 and granulocyte monocyte colony stimulating factor (GM-CSF) [33]. Both EGF and TGF-α are present in the cholesteatoma perimatrix and bind to the epidermal growth factor receptor (EGFR) [34]. Seventy-five percent of cholesteatoma keratinocytes express EGFR as opposed to only 10% of normal and canal keratinocytes [31]. TGF-α, the ligand for EGFR is also expressed across all layers of the cholesteatoma matrix [34]. Another important growth factor, which has been identified as important in cholesteatoma cells, is NF-κB. The NF-κB subunits p50 and p65 exist as inactive dimers in the cytoplasm. NF-κB is activated through nuclear translocation, which occurs upon cell insults from a variety of extracellular signals, including: cytokines, bacterial and viral products, oxidative stress, and ultraviolet light. Nuclear NF-κB binds DNA to activate the transcription of primary prosurvival and proangiogenic genes. Studies have elucidated cellular mechanisms and circumstances by which NF-κB is able to mediate changes in cellular proliferation.

Oxidative stress in cholesteatoma

The role of oxidative stress in the pathogenesis of chronic otitis media and cholesteatoma has not been fully explored yet. Aerobic organisms require ground-state oxygen to survive, but the use of oxygen during normal metabolism produces reactive oxygen species (ROS), some of which are highly toxic and deleterious to cells and tissues [35]. The most abundant ROS formed in the course of cellular metabolism is the superoxide radical ($O^{2-}\bullet$). Low-level ROSs is indispensable mediators in many cell processes including differentiation, cell cycle progression or growth arrest, apoptosis and immunity. In contrast, high doses and/or inadequate removal of ROSs result in oxidative stress, as seen in inflammation, which may cause severe metabolic malfunctions and damage to biological macromolecules [36]. The antioxidant system consists of low-molecular weight antioxidant molecules such as glutathione (GSH) and various antioxidant enzymes [37]. Paraoxonase (PON) is a high-density lipoprotein (HDL) associated antioxidant enzyme. Recent studies investigating the oxidative stress markers and antioxidant enzymes in patients with cholesteatomatous and non-cholesteatomatous chronic otitis media and in healthy subjects, revealed that the total oxidative status (TOS), and oxidative status index (OSI) were at higher levels while serum total antioxidant status (TAS), paraoxonase and arylesterase activity were lower in patients, therefore the oxidative stress and antioxidant enzyme imbalance was more severe in cases of chronic otitis media with cholesteatoma compared to the non-cholesteatoma groups [38].

Thus, the importance of oxidative stress in the pathogenesis of cholesteatoma is revealed, and the further investigation of the involved mechanisms could identify new treatment modalities.

Infection in cholesteatoma

The most serious complication of cholesteatoma is represented by bone erosion of the ossicles, otic capsule, Fallopian canal, tegmen tympani and tegmen mastoideum, which can lead to intracranial complications, conductive hearing loss, labyrinthine fistula, facial nerve paralysis, brain hernia or cerebrospinal fluid leakage [39]. Another important complication is represented by infections. The important role of infection in the pathogenesis of cholesteatoma is highlighted by the more recent identification of bacterial biofilms in otitis media and cholesteatoma [40]. Biofilms are bacterial communities enclosed in a self-produced matrix adherent to a surface. Many bacterial species relevant to otologic infections are known to form biofilms, including *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphyloccocus aureus* [41]. Bacteria within biofilms are more resistant to antibiotics and are likely responsible for the chronicity and recurrence of these infections. The presence of antibiotic-resistant bacterial biofilms in cholesteatomas may also explain their aggressiveness. Bacterial biofilms within cholesteatomas may elaborate lipopolysaccharide (LPS) and other bacterial products that stimulate osteoclastogenesis. Rayner *et al.* (1998) [42] showed that lipopolysaccharide derived from *P. aeruginosa* can induce osteoclast development in vitro and potently stimulates *in vivo* bone resorption through a toll-like receptor-4-dependent mechanism. The significance of these findings is that middle ear infection may lead to intracellular conditions that stimulate cholesteatoma epithelium to become more aggressive. Preciado *et al.* (2005) [43], showed that *P. aeruginosa* LPS, a pathologically relevant agent for cholesteatoma, was able to activate NF-κB dependent, cyclin D1 mediated keratinocyte hyperproliferation *in vitro* suggesting that middle ear infection may lead to intracellular conditions that stimulate cholesteatoma epithelium to become more aggressive.

Later studies have also elucidated the cellular mechanisms and circumstances in which NF-κB is able to mediate changes in cellular proliferation. It has been shown that NF-κB induces cyclin D1 gene expression and cellular proliferation by binding to specific sequences in the cyclin D1 promoter [44]. Other studies found the inhibitor of DNA-binding Id1 gene abundantly expressed in cholesteatoma epithelium, regulating the upstream activation of NF-κB with a subsequent activation of cyclin D1, proliferating cell nuclear antigen (PCNA) (Figure 3) and as a consequence the progression of keratinocytes in the cell cycle [45].

Proliferation markers

Cytokeratins, like CK6 and CK16 are known as intermediate filament proteins of epithelial origin and can be considered markers of keratinocytes proliferation. CK1, 13 and 19 were also found up-regulated in cholesteatoma [46]. Many authors [47] reported that the matrix of cholesteatomas expresses CK16 on suprabasal layers, emphasizing that the expression of this protein filament characterizes a hyperproliferative epithelium. Vennix *et al.* [48] analyzed the pattern of cytokeratins and suggested that the cholesteatoma matrix is not a result of a metaplastic change. In the study, they found an epithelium similar to

that of the tympanic membrane and to the skin of the external auditory canal, but in different stages of proliferation, depending on the level of inflammation present. Kim *et al.* [49] showed an increased expression of cytokeratin 13 and cytokeratin 16 in the peripheral area of the pars tensa of cholesteatoma induced by ear canal ligation and in the peripheral and central area of pars tensa of cholesteatoma induced by Eustachian tube obstruction.

Cholesteatoma is a disorder of uncoordinated proliferation, migration and invasion of the involved keratinocytes. The epithelial proliferation marker Ki67 (Figure 4) and also the neutrophil gelatinase-associated lipocalin (NGAL) expression is usually higher in cholesteatoma tissues compared to controls [50, 51].

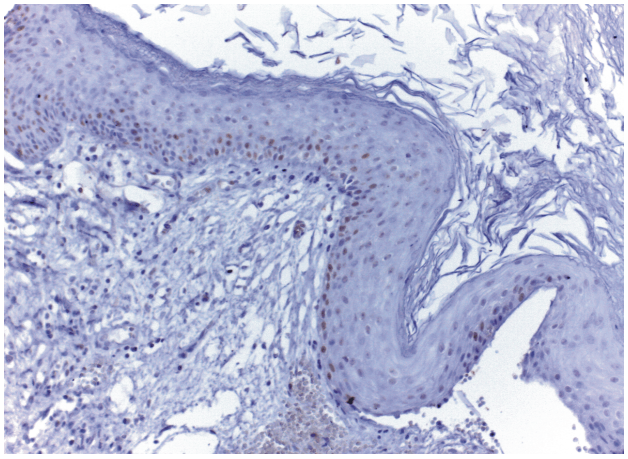

Figure 3 – ***Pathologic specimen of acquired cholesteatoma. Cholesteatoma epithelium shows proliferating cell nuclear antigen (PCNA), ×100.***

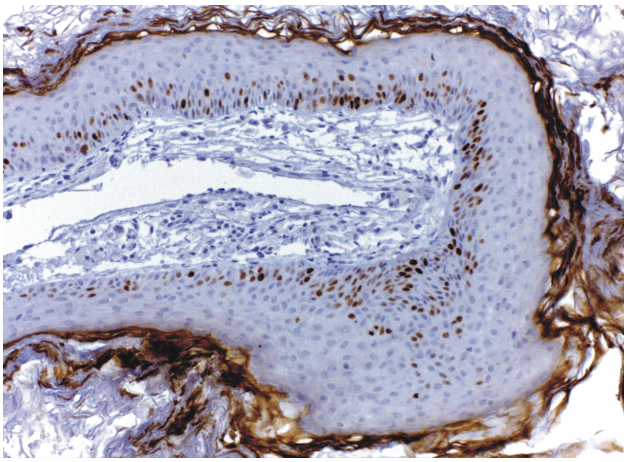

Figure 4 – ***Pathologic specimen of acquired cholesteatoma. Cholesteatoma epithelium shows the epithelial proliferation marker Ki67, ×100.***

Pediatric cholesteatomas are usually more aggressive and invasive, having a higher expression of PCNA and MIB1 (which recognizes a nuclear antigen expressed by cells) compared to adult forms [52]. Also implicated in the differentiation, proliferation and apoptosis of keratinocytes in cholesteatoma are c-jun protein (transcription factor), p53 (negative regulator of proliferation that induces DNA damage) [53], p63 (p53 homologue) and survivin (inhibitor of apoptosis). Macrophage Inhibitor Factor (MIF) is correlated with the aggressiveness level in cholesteatomas and the recurrence of the disease [54]. Recent concepts assume that cholesteatoma might be a disturbed wound-healing process [55], often with an underlying inflammatory tissue repair reaction [56]. It has been hypothesized that the development of cholesteatoma involves an altered control of cellular proliferation, which affects the balance towards the aggressive and invasive growth of squamous epithelium [57]. However, it is yet unclear whether this altered control is due to defects in the mechanisms and underlying genes that control proliferation, or to cytokines released from infiltrating inflammatory cells.

Studies have demonstrated that the cholesteatoma tissue expresses tumor-relevant genes normally expressed in chronically inflamed tissue. Moreover, tumor suppressor genes CDH18, 19 and ID4, PAX3, LAMC2 and TRAF2B, which are known to be down regulated in various tumors [58–60], are also down regulated in cholesteatoma. Genes like CEACA6, MMPs and their inhibitor RECK [61], as well as SPRRB2 are up regulated.

Genes who were important for inflammation, for example, KRT6B, SPP1, and S100A7A are highly up regulated in cholesteatoma compared to external auditory skin. However, further studies should evaluate a potential link between inflammation, up-regulation of tumor-related transcripts and cholesteatoma-pathogenesis in more detail [62].

Similarities were found between gene expressions of tumors and cholesteatoma, which could explain the aggressiveness and local destruction in cholesteatoma cases compared with otitis media with no cholesteatoma, even if the inflammatory process is similar in both conditions. Nevertheless, there are no hints for inherent genetic instability.

Conclusions and future perspectives

Cholesteatoma may be considered a cell growth disorder, encompassing a number of complex and dynamic events, which involve cellular and extracellular components; its growth requires angiogenesis in the perimatrix connective tissue, and the substances present in the healing cascade may play an important role in its growth and development. However, it is still unknown whether this lack of control is caused by defects in genes that control proliferation, by the cytokines released by inflammatory cells or by other, still unknown, mechanisms.

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