INVITED REVIEW

Different Aspects of Cholesteatoma Pathogenesis in Own Research

Ewa Olszewska

Department of Otolaryngology Medical University of Bialystok, Poland

Cholesteatoma is a cyst-like structure composed of keratinizing squamous epithelium (matrix) that contains epithelial keratin derbis and a subepithelial connective tissue. The pathogenesis of cholesteatoma remains still not completely understood. We can focus on two pathogenetic concepts of congenital and acquired cholesteatoma: 1. What is the origin of keratinizing squamous epithelium in cholesteatoma? and 2. what factors are involved in such an invasive and hyperproliferative behavior of cholesteatoma?

Based on own research and literature I will due the discussion to the different aspects of cholesteatoma pathogenesis: activity of selected enzymes in cholesteatoma tissue, proliferation and apoptosis of keratinocytes within cholesteatoma matrix and the development of new blood vessel formation in cholesteatoma tissue.

The final conclusions are following: the cytokeratin distribution pattern is identical in congenital and acquired cholesteatoma; the proliferation of keratinocytes seems to be associated with increased keratinocyte migration in certain sites within cholesteatoma epithelium; the increased rate of keratinocyte death in cholesteatoma may be related to apoptosis. The process may be responsible for the differentiation and accumulation of keratin derbis within middle ear; enchanced vascularization in cholesteatoma causes a continuous and pathologic growth of cholesteatoma mass; catabolic reactions involving glycoproteins, glycolipids and proteoglycans may play a role in cholesteatoma-related bone resorption.

Therapeutic strategies that act to inhibit selected enzymes and proliferating factors as well as to promote apoptitic agents may serve as a useful adjunct in the treatment of cholesteatoma.

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Since Du Verney gave the first description of cholesteatoma-like mass, which he called "steatoma" in 1683, numerous investigations of chronic middle ear with cholesteatoma have been conducted [1]. The question how this knowlegde can be useful in the clinical treatment of cholesteatoma is still open. The tendency of cholesteatoma to bone erosion and the lack of effective, nonsurgical treatment underline the importance of investigating the pathomechanisms of this disease. After years of studying those mechanisms and searching for the pathogenic factors, the only treatment for cholesteatoma is surgery, and its prevention is still not completely known. I am puzzled with questions what the first signal is to start cholesteatomous development, what signals are triggered a change with controlled retraction pocket towards cholesteatoma, what presses keratinocytes into proliferation and what possibilities we have to discover alternative treatment of cholesteatoma and prevention of its development or reccurence.

According several studies on cholestetoma histology we well know that congenital as well as primary and secondary acquired cholestetomas have similar histology. It is a cyst-like structure composed of keratinizing squamous epithelium (matrix) that contains epithelial keratin derbis and a subepithelial

Corresponding address:
Ewa Olszewska
Ul. Sklodooskiej 24 A 15-256 Bialystok, Poland
Sponsored by grent number 40107931/1821 from Ministry of Science and Education, Poland
Phone: +48 85 7468269; Fax. +48 85 7468697; E-mail: ewaolsz@yahoo.com

connective tissue. The pathogenesis of cholesteatoma remains still not completely understood. We can focus on two pathogenetic concepts of congenital and acquired cholesteatoma: 1. What is the origin of keratinizing squamous epithelium in cholesteatoma? and 2. What factors are involved in such an invasive and hyperproliferative behavior of cholesteatoma?

In the microenvironment of cholesteatoma the following processes are observed: inflammation, keratinocyte prolifeartion and apoptosis, angiogenesis and bone resorption.

Bone resorption is a basic biologic process found in physiological and pathological conditions in humans. It is well accepted that bone remodeling and bone resorption are caused by the local activity of osteoclasts. The osteoclast is a specific macrophage created by the differentiation of monocyte and macrophage precursor cells at the bone surface. The mature osteoclast is activated by signals which leeds to initiation of bone remodeling [2]. Bone resorption observed widely in cholesteatoma occurs as a result of induction of various cytokines and enzymes through an inflammatory reaction in the subepithelial tissue of cholesteatoma. During inflammation a variety of factors such as: IL-1α, IL-1β, TNF-α and interferon β are released. Recently, it has been shown that bone remodelling and bone loss are controlled by a balance between 2 other factors namely: by the receptor activator of RANK and its ligand RANKL [3]. RANKL can activate mature osteoclasts in vitro and can lead rapidly to the resorption of bone in vivo by activating pre-existing osteoclasts. The close contact between stroma and bone marrow cell types was essential for osteoclastogenesis. It is now know that this system allowed for production of two haematopoietic factors that are both necessary and sufficient for osteoclastogenesis: previously mention RANKL and M-CSF. An important moment for understanding the whole process of osteoclastogenesis was the identification of osteoprotegerin (OPG) - a soluble TNFR-related protein that blocks osteoclasts formation in vitro and bone resorption in vivo [4].

Several families of enzymes have been demontrated to play a pivotal role in controlling the behavior of cholesteatoma. Many bone matrix proteins are glycoconjugates. Enzymes capable of degrading extracellular matrix components and extracellular bony tissue are among those that lead to the resorption of bone. The group of enzymes which significance in the process of bone resorption is not completely known, are lysosomal exoglycosidases. The family of exoglycosidases, has been only marginally considered in cholesteatoma and the lack of interest in catabolism of glycoproteins is surprising in light of the fact that
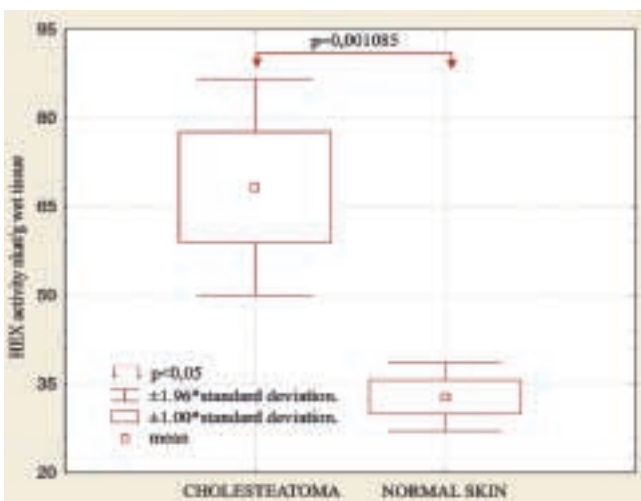

Figure 1. Hexosaminidase activity in cholesteatoma tissue compared with skin homogenates. Parametric statistics using Student's t test.
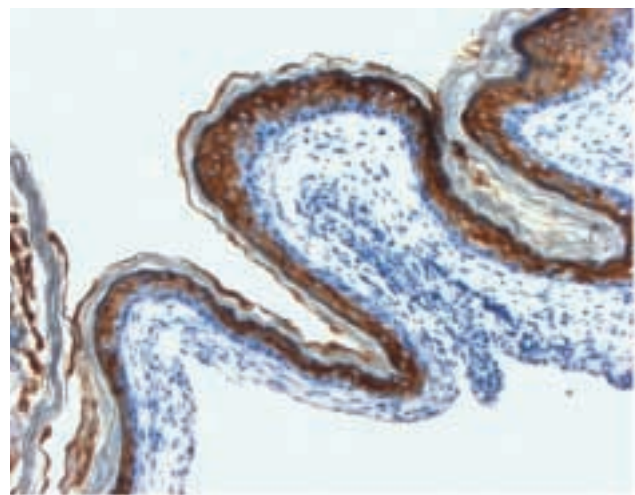

Figure 2. Cholesteatoma. The expression of CK10 shows strong positive immunohistochemical reaction within the basal and suprabasal layers of cholesteatoma epithelium. A labeled streptavidin peroxide method. Magnification x200.

most bone matrix macromolecules are glycosylated. A high level of carbohydrates has been demonstrated. The carbohydrates may significantly affect the proteolytic cleavage of the extracellular bone matrix adjacent to cholesteatoma. Therefore, we made an attempt to investigate the markers of a catabolic process associated with chronic inflammatory state.

We assessed the level of catabolism of glycoconjugates in assays of cholesteatoma extracts, quantifying 3 lysosomal exoglycosidases: N-acetyl-β-D-hexosaminidase (HEX), β-galactosidase (GAL) and α-mannosidase (MAN). Among them the highest activity is demonstrated by N-acetyl- β-D-hexosaminidase. HEX catalyzes the release of terminal sugar moieties from non-reducing ends of oligossacharide chains of N-acetyl-β-D-glucosamine and N-acetyl-β-D-galactosamine in glycoproteins, glycosaminoglycans and glycolipids [5]. Lysosomal exoglycosidases are synthesized in the form of precursors and transported from their site of synthesis on ribosomes associated with the rough endoplasmic reticulum to the lysosomes. Lysosomal exoglycosidases play an intermediatory role in the chronic inflammatory process. Controlled observations indicate that exoglycosidases are up-regulated in rheumatoid arthritis, idiopathic juvenile arthritis and osteoarthritis [6].

There are several different cells which are able to release HEX under inflammatory conditions. During inflammation the stimulation of fibroblasts with Il-1β results in an increase in secretion of HEX. It has been reported a marked increase in HEX release in response to direct contact of mast cells with activated T cell membranes. HEX is released after four hours of incubation with activated T cell membranes and increases over time. HEX is produced in and released from leukocytes, neutral granulocytes, mast cells and fibroblasts [7]. Most of these cells are found in cholesteatoma subepithelial connective tissue.

To assess exoglycosidases activity, release of p-nitrophenol from p-nitrophenol derivates of substracts, α-mannose and β-galactose was used. We observed a significantly higher activity of HEX in cholesteatoma tissue compared with that in normal skin. The mean release of HEX from activated cells was about 70 nkat/g wet tissue in cholesteatoma homogenates and about 32 nkat/g wet tissue in skin specimens. We also found that the positive correlation between two variables, the enzyme activity in normal skin (X) and the enzyme activity in cholesteatoma (Y), has been performed to prove the functional relationship. This means that each value of the independent variable X, meets only one particular value of the dependent varable Y. We obtained a positive correlation: r>0.3 [8]. The mean activity of α-MAN was 1.76 nkat/g wet tissue of cholesteatoma and 0.61 nkat/g wet tissue in skin. (Figure 1) The correlation of two variables
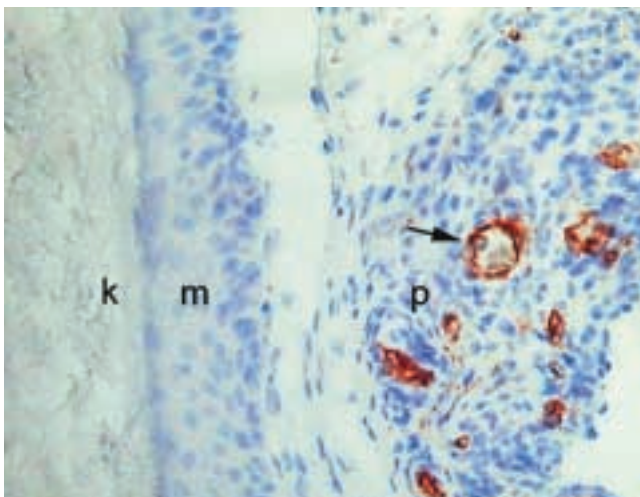

Figure 3. Cholesteatoma. Streptoavidin peroxide metod with anti-CD34. Small brown-stained vessels within perimatrix. x200.
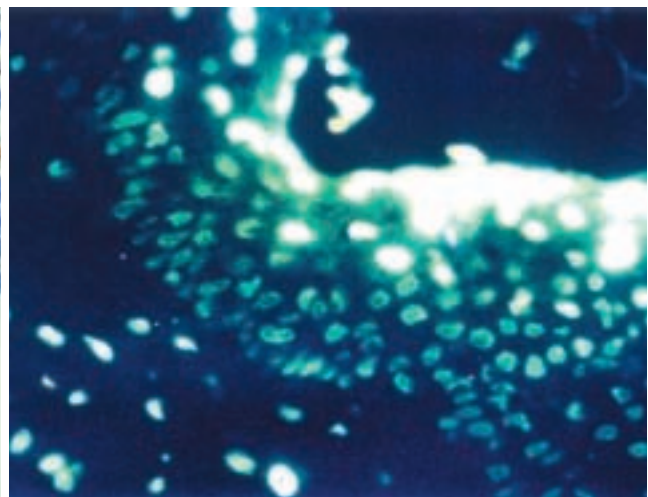

Figure 4. Numerous apoptotic cells in cholesteatoma epithelium. TUNEL metod. Magnification x400.

(MAN activity in cholesteatoma and MAN activity in a normal skin specimens) was also positive: r>0.4. The mean activity of β-GAL from cholesteatoma cells was 1.8 nkat/g wet tissue and 0.8 nkat/gwet tissue in skin. Pearson's coefficient was >0.5 and proved that the correlation is strongly positive [9].

Morphologically, cholesteatoma is characterized by the presence of keratinizing stratified squamous epithelium accompanied by an inflammatory reaction of the subepithelial connective tissue. As soon as the epithelium begins to hyperproliferate the destructive behavior of cholesteatoma is triggered. Hyperproliferative characteristics have been evidenced by the expression of cytokeratins, Ki67 antigens, PCNA etc. Cytokeratins are released by macrophages, lymphocytes and other cells at the site of infection. They are mediators of inflammation and the immune response. The immune respons is critical for the primary defense against middle ear infection. Immunohistochemical processing was performed using the labelled streptavidin peroxidase metod to demonstrate the presence of selected cytokeratins in cells of human epidermis and congenital and acquired cholesteatoma tissues. Positive reaction was obtained for CK4, 34βE12, CK10 and CK14 both in cholesteatoma and skin specimens. However, more extensive positive reaction with those CKs was observed in cholesteatoma epithelium. Cholesteatoma and skin revealed no positive reaction with anti CK19. Generally, the pattern of cytokeratins both in congenital and acquired cholesteatoma was similar [10].

Angiogenesis is a multiple stage process leading to the formation of a new blood vessel. It can occur in physiological processes, e.g. wound healing and pathological ones such as inflammation and cancer. In cholesteatoma, angiogenesis appears when proliferating epithelial cells are increased metabolic demands. In the response to inflammation in cholesteatoma perimatrix, an intensification of new blood vessel formation is observed. Monocytes, macrophages, leukocytes produce cytokines such as: fibroblasts growth factor, vascular endothelial growth factor, transforming growth factorα, therefore angiogenesis is induced by inflammatory cells. But there also another way by which angiogenesis in cholesteatoma is stimulated, namely by keratinocytes which are able to release some of the angiogenic factors. The increased release of angiogenic factors may initiate angiogenesis which results in the development of immature blood vessels and favours the destructive activity of cholesteatoma. For our study, we used cholesteatoma collected from adult patients. Monoclonal mouse antibodies agains human CD34 antigens were used for immunohistochemistry which was performed using a standard avidin-biotin complex technique. CD34 is a protein localized on small-vessel endothelial cells and precursor myeloidal cells. It appears at the early stage of formation and differentiation of new vessels as well as play a role in the regulation of epithelial cell migration during the vessel maturation. We observed significantly more intensive process of angiogenesis in cholesteatoma compared with that in normal skin specimens. The predominance of small blood vessels leeds to the formation of microcirculation in middle ear [11]. (Figure 3)

In cholesteatoma, keratin derbis is cumulated as a result of an increase in the percentage of dead cells formed during the differentiation of keratinocytes. In our study, cholesteatomas were collected during surgical procedures of the ear. Normal skin specimens taken from retroauricular skin served as controls. We observed apoptosis of the epithelial cells by means of antibody against APO2.7 antigen, a protein localized on the mitochondria membrane that appears in the early stage of apoptosis. The membstain Apoptosis kit Direct based on in situ labeling of nuclear DNA fragmentation (Tolt-mediated dUTP Nick and labeling TUNEL staining) was used. Apoptotic cells were found in the suprabasal layers of cholesteatoma epithelium (47.39±6.2%) and the normal epidermis (28.5±8.1%) [12]. However we need p51 and p21 study for further evaluation. (Figure 4)

After many years of research on inflammatory mediators in cholesteatoma, there are still no satisfying answers to the following questions: what is the state of our knowledge and how we can use it for patient treatment? What we already know according our studies are:

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1. The cytokeratin distribution pattern is identical in congenital and acquired cholesteatoma;

2. The proliferation of keratinocytes seems to be associated with increased keratinocyte migration in certain sites within cholesteatoma epithelium;

3. The increased rate of keratinocyte death in cholesteatoma may be related to apoptosis. The process may be responsible for the differentiation and accumulation of keratin derbis within middle ear;

4. Enchanced vascularization in cholesteatoma causes a continuous and pathologic growth of cholesteatoma mass;

5. Catabolic reactions involving glycoproteins, glycolipids and proteoglycans may play a role in cholesteatoma-related bone resorption. Therapeutic strategies that act to inhibit lysosomal exoglycosidases may serve as a useful adjunct in the treatment of cholesteatoma.

Acknowledgments

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