Catabolism of Glycoconjugates in Chronic Otitis Media with Cholesteatoma

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Chronic ear disease with cholesteatoma is characterized by an intrusion of keratinizing stratified squamous epithelium into the middle ear manifesting bone resorption at the interface of the perimatrix. The aim of our study was to investigate the markers of a catabolic process associated with several chronic inflammatory states. We assessed the level of catabolism of glycoconjugates in assays of cholesteatoma extracts, quantifying two lysosomal exoglycosidases: α-mannosidase (α-MAN) and β-galactosidase (β-GAL). Cholesteatomas (n = 15) and normal adult postauricular skin served as controls (n = 15) were collected from the patients during surgery owing to chronic otitis media. To assess exoglycosidase activity, release of p-nitrophenol from p-nitrophenol derivatives of α-mannose and β-galactose was used. In 13 of 15 specimens, we observed significantly higher activity of investigated enzymes in cholesteatoma tissue compared with control tissue (postauricular skin). The mean activity of α-MAN from the cholesteatoma cells was 1.76 ± 1.10 nkat/g wet tissue and 0.61 ± 0.21 nkat/g wet tissue in the control probes. The mean activity of β-GAL from the cholesteatoma cells was 1.77 ± 1.07 nkat/g wet tissue and 0.87 ± 0.20 nkat/g wet tissue in the control probes. Catabolic reactions involving glycoproteins, glycolipids, and proteoglycans may play a role in cholesteatoma-related bone resorption. The present data indicating that the lysosomal exoglycosidases α-MAN and β-GAL are significantly and consistently elevated suggest the need to further correlations assessment between levels of α-MAN and β-GAL and cholesteatoma behavior. Further research should also evaluate the relative importance of these particular exoglycosidases in manifesting bone resorption in considering the spectrum of identified inflammatory mediators.

Key words: α-mannosidase, β-galactosidase, catabolism of glycoconjugates, cholesteatoma, normal postauricular skin

In cholesteatoma, the major clinical symptoms are caused by chronic inflammation and bone resorption. Inflammation is induced by an immune response to tissue injury. In cholesteatoma, the inflammatory response may result in pathologic alteration of tissue behavior. Cholesteatoma pathogenesis can be interpreted as the reaction of migrating epithelium to an ongoing inflammation and bacterial infection. The inflammatory granulation tissue often appears along the invading epithelium in active cholesteatoma. The exact molecular mechanisms of osteolysis of the temporal bone during the course of chronic inflammation in cholesteatoma have not been completely understood.[1] Cholesteatoma, an epidermal cyst, is a destructive lesion of the temporal bone that gradually expands and causes complications by erosion of the adjacent bony structures. Bone resorption can result in destruction of the ossicular chain and otic capsule, with consecutive hearing loss, vestibular dysfunction, facial paralysis, and intracranial complications. Resorption of bone occurs generally in a localized area bordering the cholesteatoma perimatrix and is mediated by osteoclasts.[2] The perimatrix layer of cholesteatoma that is subepithelial connective tissue shows the activities of different enzymes. Among them are lysosomal exoglysosidases, the activities of which are significantly higher in cholesteatoma compared with the activity in normal skin specimens. We observed the ossicular damage owing to the enzyme and osteoclast activities.[3] It is

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Supported by grant number 401 079 31/1821 from the Ministry of Science and Education, Poland.

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Journal of Investigative Medicine 2007;55:248–254.

DOI 10.2310/6650.2007.00005

well accepted that bone remodeling and bone resorption are caused by the local activity of osteoclasts.[2] 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 leads to initiation of bone remodeling.[4] Inflammatory mediators such as interleukin (IL)-1α, IL-1β, and tumor necrosis factor α and growth factors released in cholesteatoma may initiate osteoclast recruitment and bone resorption.[5] Several families of enzymes have been demonstrated to play a pivotal role in controlling the behavior of cholesteatoma. Many bone matrix proteins are glycoconjugates. Among them there are mannosidases, galactosidases, fucosidases, and hexosaminidases. The enzymes 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.[6] Enzymes capable of degrading extracellular matrix components of skin and extracellular bony tissue are among those that lead to resorption of bone. Lysosomal proteolytic enzymes manifesting tissue destruction are considered to be of great significance in cholesteatoma. Those enzymes are supposed to be produced by cholesteatoma and may also cause destruction of the neighboring tissues. Proteases such as matrix metalloproteinases and cathepsins likely play an important role in the degradation of the osteoid layer.[7]

Lysosomal exoglycosidases, such as mannosidases and galactosidases, play an intermediary role in the chronic inflammatory process. Controlled observations indicate that exoglycosidases are up-regulated in rheumatoid arthritis and juvenile idiopathic arthritis.[8] These data provide clues as to the suspected proteins that induce bone destruction in cholesteatoma.

Lysosomal α-mannosidase (α-MAN; EC 3.2.1.24) is an exoglycosidase that cleaves α-linked mannose residues from the nonreducing end during the ordered degradation of N-linked glycoproteins.[9] Therefore, its activity may associate with the intensity of catabolism of N-linked oligosaccharide chains of glycoproteins. α-MAN is extractable from human liver, fibroblasts, and other tissues.[10]

β-Galactosidase (β-GAL; EC 3.2.2.23) is a glycoside hydrolase localized in the lysosome.[11,12] It catalyzes the hydrolysis of terminal β-glycosidic bonds present in oligosaccharide chains of glycolipids, glycoproteins, and glycosaminoglycans.[13] Therefore, its activity may reflect the intensity of catabolism of oligosaccharide chains containing galactose, that is, glycosaminoglycans, glycoproteins, and glycolipids. β-GAL has been taken as an indicator for lysosomal enzyme release.[14] The enzyme is capable of degrading extracellular matrix components, mainly glycosaminoglycans and glycoproteins. A pH around 5.0 exists inside of lysosomes.[15] The activity of the enzyme is maximal at acid pH 3 to 5.[12]

Although exoglycosidases have been shown to manifest tissue degradation in different diseases, their activity levels in cholesteatoma have not received previous formal assessment. For the first time, we investigated the activities of α-MAN and β-GAL in cholesteatoma tissue, performing identical, blinded assays in normal skin biopsies.

Material and Methods

All procedures were approved by the Medical University of Bialystok Investigational Review Board, and patients gave consent for matched-skin biopsies in addition to the planned otologic procedure. Human cholesteatoma ($n = 15$) and normal retroauricular skin from a region deemed to be uninflamed and located approximately 2 cm posterior to the auricular fold ($n = 15$) were harvested and immediately frozen at $-80^{\circ}C$ from the same patients during the surgical procedures owing to chronic otitis media. The age of the patients ranged between 35 and 69 years (mean age 42.3 years). The history of chronic otitis media ranged from 6 months to 5 years. Four cholesteatomas were classified as primary acquired cholesteatoma and 11 as secondary acquired cholesteatoma. Granulation tissue and purulent otorrhea were present in 10 cases.

To identify osteoclasts, we perform hematoxylin-eosin staining (Figure 1). Two independent observers, blinded to the experimental procedure, identified osteoclasts in the area adjacent to the ossicles. The following criteria, in accordance with Chole and colleagues,[16] have been assumed: (a) a ruffled border, (b) granular cytoplasm, (c) large multiple-nucleate (≥ 2) tartarate-resistant acid phosphatase (TRAP)-positive cells; and (d) the absence of a lamina limitans along a surface of the bone without an organelle and an organelle-rich opposite region.

A subsequent classification was also made, according to Tos,[17] based on the site of cholesteatoma origination: attic cholesteatoma ($n = 2$), sinus cholesteatoma ($n = 9$), and tensa cholesteatoma ($n = 4$).

Preparation of Homogenate

Cholesteatoma and skin specimens were thawed out and weighed. Specimens were washed with 0.9% NaCl to remove leukocytes and fragments of mucosa.

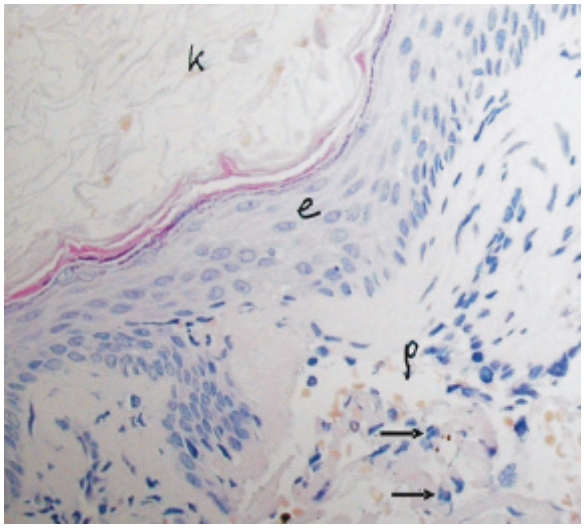

Figure 1 The histology of cholesteatoma (hematoxylin-eosin stain; ×200 original magnificaiton) revealing osteoclasts adjacent to the ossicle (*arrows*). e = matrix of cholesteatoma; k = keratin debris; p = cholesteatoma perimatrix.

Specimens were suspended in 0.05 M citrate-phosphate buffer at pH 4.3 at a 1:9 ratio (w/v) and homogenized for 2 minutes using homogenizer Ultra-Turrax T8 (JKA, Werke GmbH & Co., KG, Haufen, Germany). Homogenates were then centrifuged for 30 minutes (12,000*g*) at 4°C. Supernatant was stored at −70°C for further studies.

Reagents

p-Nitrophenyl-α-MAN and p-nitrophenyl-β-GAL were obtained from Sigma (St. Louis, MO) and other reagents from Polish Chemical Reagents (Gliwice, Poland).

α-MAN and β-GAL Release and Assay

The activity of α-MAN and β-GAL in cholesteatoma and skin homogenates was determined by the method of Chatterjee and colleagues,[18] adopting the modifications of Zwierz and colleagues.[19] These methods offer a robust method of quantifying the activity levels of exoglycosidase established in the unit of nkat per gram wet tissue.

The enzyme activity within the cholesteatoma and skin samples was statistically compared with analyses conducted using the *STATISTICA* program (StatSoft, Tulsa, OK). Comparisons were made with the Wilcoxon matched pairs test; differences at the $p < .05$ level were considered significant.

Results

In 13 of 15 specimens, we observed significantly higher activity of α-MAN in cholesteatoma tissue compared with that in normal postauricular skin. The mean activity of α-MAN was 1.75-fold higher compared with that of controls. In three cholesteatoma specimens, the activity of α-MAN was 4.01- to 4.77-fold higher than in the skin. In six cholesteatoma specimens, the activity of α-MAN was 3.10- to 3.99-fold higher than in the skin; in one specimen, it was 2.04-fold higher; and in three specimens, it was 1.5- to 1.79-fold higher.

In two cholesteatoma specimens, the activity of α-MAN revealed small decrements relative to control tissue (0.22 and 0.35 in cholesteatoma/0.94 and 0.418 in the skin).

The levels of MAN activity ranged from 0.22 to 4.152 nkat/g wet tissue of cholesteatoma. In normal postauricular skin, the level of MAN activity ranged from 0.35 to 1.158 nkat/g wet tissue (Figure 2). The mean activity of MAN from the cholesteatoma cells was 1.76 ± 1.10 nkat/g wet tissue. In skin specimens, the mean activity of MAN was 0.61 ± 0.21 nkat/g wet tissue. As data have nonparametric measurements, we used a Wilcoxon signed rank test.

The correlation of two variables (MAN activity in cholesteatoma and MAN activity in a normal skin specimen) is shown in Figure 3. Pearson's coefficient is equal $r = .48955$ and proved that the correlation is average positive.

In 11 of 15 specimens, we observed significantly higher activity of β-GAL in cholesteatoma tissue compared with that in normal postauricular skin. The mean activity of β-GAL from cholesteatoma cells was 1.77-fold higher compared with controls. In three

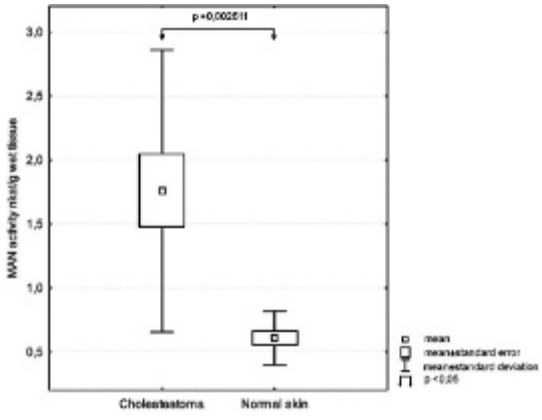

Figure 2 Activity of α-mannosidase (MAN) in cholesteatoma (statistical differences).

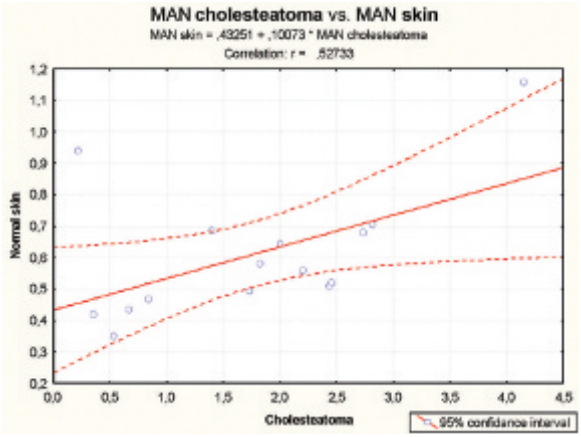

Figure 3 Distributions of α-mannosidase (MAN) activity levels in cholesteatoma and normal postauricular skin. Wilcoxon matched pairs test. Pearson's coefficient is positive ($r = .48955$).

cholesteatoma specimens, the activity of β-GAL was 3.08- to 3.40-fold higher than in the skin. In eight cholesteatoma specimens, the activity of β-GAL was 2.00- to 2.94-fold higher than in the skin.

In two cholesteatoma specimens, the activity of β-GAL revealed small decrements relative to control tissue (0.23–0.852 in cholesteatoma and 0.81–1.208 in the skin).

The levels of GAL activity ranged from 0.234 to 4.014 nkat/g wet tissue of cholesteatoma. In normal postauricular skin, the level of GAL activity ranged from 0.668 to 1.278 nkat/g wet tissue (Figure 4). The mean activity of GAL from the cholesteatoma cells was 1.77 ± 1.07 nkat/g wet tissue. In the control group, the GAL activity was 0.87 ± 0.20 nkat/g wet tissue.

Figure 5 described the correlation of two variables (GAL activity in cholesteatoma and GAL activity in a normal skin specimen). Pearson's coefficient is equal $r = .52733$ and proved that the correlation is strongly positive.

Discussion

Bone resorption observed widely in cholesteatoma occurs as a result of induction of various cytokines and enzymes through an inflammatory reaction in the epithelium and subepithelial granulation tissue of cholesteatoma.[20,21] Histopathologically, cholesteatoma is a cystic lesion lined by keratinizing squamous epithelium resting on connective tissue, including scattered fibroblasts and collagen infiltrated by lymphocytes and macrophages.[22,23] The tissue adjacent to the cholesteatoma revealed the presence of macrophages, giant cells, and lymphocytes around keratin debris.[24] These findings suggest that the inflammmatory response is characterized by lymphocyte infiltration.[25]

Degenerated keratin protein in cholesteatoma is engulfed by macrophages, which play a pivotal role in cell-mediated immunity. It is possible that activated macrophages, assisted by lymphokines, generate active oxygen species, various hydrolytic enzymes, and cytokines at the inflammatory site and may subsequently cause bone resorption.[25]

The family of exoglycosidases, however, has been only marginally considered in chronic inflammatory

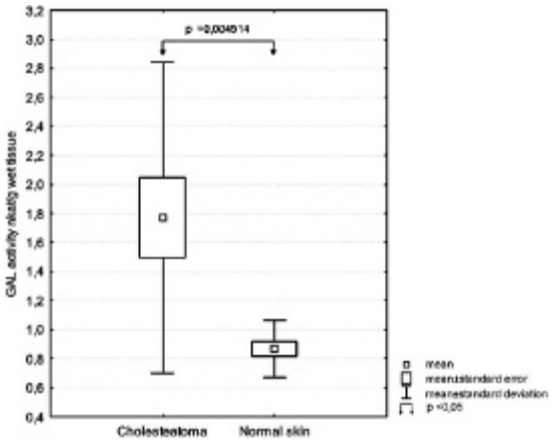

Figure 4 Activity of β-galactosidase (GAL) in cholesteatoma (statistical differences).

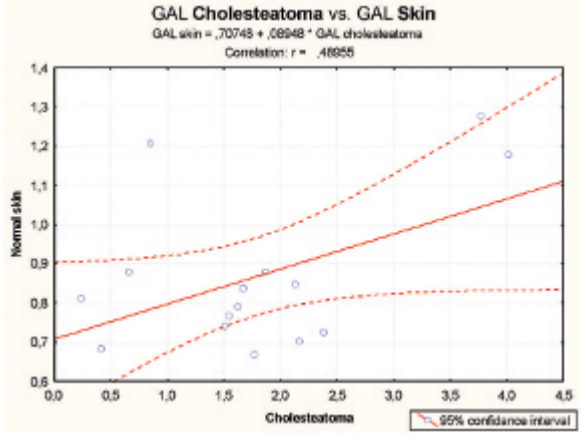

Figure 5 Distributions of β-galactosidase activity levels in cholesteatoma and normal postauricular skin. Wilcoxon matched pairs test. Pearson's coefficient is strongly positive ($r = .52733$).

diseases such as rheumatoid arthritis and juvenile idiopathic arthritis.[8] Our study is the first one to address the question of exoglycosidase activity in cholesteatoma. The lack of interest in catabolism of glycoproteins is surprising in light of the fact that 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 the cholesteatoma. Mannosidases are synthesized as single-chain precursors that are proteolyzed into five polypeptides. The significantly higher activity of lysosomal exoglycosidases compared with control groups has been proved in different diseases, such as rheumatoid arthritis, chronic *Borrelia* arthritis, and juvenile idiopathic arthritis.[8,26]

In cholesteatoma, the major clinical symptoms and disability of patients are caused by an irreversible destruction of bone adjacent to the cholesteatoma perimatrix. Enzymes capable of degrading extracellular matrix components, such as collagen, and exposing keratinocytes to cytotoxic and apoptotic factors are considered to be the major effector molecules in bony degradation. The presence of many enzymes in osteoclasts has been confirmed. The most important for their osteolytic activity are cathepsins D and K, metalloproteinase 9, reduced nicotinamide adenine dinucleotide phosphate oxidase, and lysosomal exoglycosidases.[27,28]

In the literature, it has been demonstrated that α-MAN is extracted from human fibroblasts. Fibroblasts play a role in hyperkeratosis of middle ear cholesteatoma by releasing molecules involved in inflammation and epidermal growth.[29] A proliferating phase of cholesteatoma is also characterized by fibroblasts. In our previous study, we demonstrated the increased activity of one of the exoglycosidases, *N*-acetyl-β-D hexosaminidase (HEX), in cholesteatoma tissue.[27] The increase in HEX activity was observed in several inflammatory diseases, such as rheumatoid arthritis, idiopathic juvenile arthritis, osteoarthritis, and chronic glomerulonephritis.[30–33] Owing to the essential role of HEX in inflammatory diseases, it may be assumed that the significance of α-MAN and β-GAL as catabolic enzymes is also crucial in the pathogenesis of cholesteatoma. α-MAN may indicate the catabolism of N-linked glycoproteins, glycolipids, and proteoglycans. We demonstrated, for the fist time, that α-MAN and β-GAL are present in the cholesteatoma specimens. α-MAN may indicate the catabolism of N-linked glycoproteins. β-GAL may be considered as an indicator of the catabolism of glycoproteins, glycolipids, and proteoglycans. The activity of α-MAN and β-GAL was found to be significantly increased in cholesteatoma tissue compared with controls. In cholesteatoma, the mean activity of α-MAN was 1.75-fold and the mean β-GAL activity was 1.77-fold higher compared with control tissue. 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 variable, Y. The correlation between variables X and Y is the measure of the linear force of these variables. On the diagram, points corresponding to particular values create the correlating scatter diagram (see Figures 3 and 5). It is accepted that you obtain the positive correlation when the elevated value of one variable complies with an elevation of the mean value of the second variable. The most common coefficient is the linear Pearson's coefficient, which is determined as r_{XY}. The correlation was an average positive when r_{XY} is more than 0.3 and less than 0.5 and strongly positive when r_{XY} is more than 0.5 and less than 0.7.

After 30 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 can we use it for patient treatment? Secretory leukocyte protease inhibitor contributes to host protection against inflammatory cell and destructive enzymes in the chronic inflammatory state of cholesteatoma.[34] Huisman and colleaguges demonstrated increased levels of involucrin and increased levels of the activated forms of p38 and extracellular signal-regulated kinases in cholesteatoma epithelium.[35] The authors discussed whether this increase is part of an inflammation response in cholesteatoma. It is generally accepted that cytokines and epidermal growth factor are mediators in the destructive behavior of cholesteatoma and result in keratinocyte activity.[36] What we know now about catabolic reactions involving glycoproteins, glycolipids, and proteoglycans is that these reactions may play a role in cholesteatoma-related bone resorption. The lysosomal exoglycosidases α-MAN and β-GAL are significantly and consistently elevated and suggest the need to further correlations assessment between levels of α-MAN and β-GAL and cholesteatoma bone resorption. Further reasearch should also evaluate the relative importance of these particular exoglycosidases in manifesting bone resorption in considering the spectrum of identified inflammatory mediators. Therapeutic strategies that act to inhibit α-MAN and β-GAL activity may serve as a useful adjunct in the treatment of cholesteatomas.

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