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The significance of the expression of cell proliferation and inflammation markers in the development of acquired middle ear cholesteatoma

Značaj ekspresije markera ćelijske proliferacije i inflamacije u razvoju stečenog holesteatoma srednjeg uva

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Abstract

Background/Aim. Permanent proliferation and periodical infection are the main clinical characteristics of acquired middle ear cholesteatoma. The aim of this study was to research immunohistochemical characteristics of the skin along with the cholesteatoma process in the nearby tissue. This research should influence further studying of etiology and development of acquired middle ear cholesteatoma. **Methods.** We investigated clinical, histological and immunohistochemical characteristics of cholesteatoma in 50 samples from operated patients with acquired middle ear cholesteatomas. We classified all samples according to their clinical characteristics of cholesteatoma such as bone destruction, presence of infection or cholesteatoma extension and histological characteristics of cholesteatoma such as keratinisation, inflammatory process and extracellular matrix proliferation. We used mouse monoclonal antibodies for proliferating cell nuclear antigen (MAbs for PCNA), Ki-67, COX-2, CD 4 and CD 8 lymphocytes to investigate the expression of those characteristics in the cholesteatoma and in the control skin tissue. Statistical analyses were performed using SPSS for Windows version 16.0 (SPSS, Chicago, IL, USA). We used the independent group *t*-test, Spearman's correlation analysis and Mann-Whitney U test to analyze statistical analysis. **Results.** Expression of PCNA, Ki-67, COX-2 and CD 8 lymphocytes in more serious clinical picture of cholesteatoma was almost equal as in less serious clinical picture of cholesteatoma. There was statistically significantly higher concentration of inflammation marker CD 4 lymphocytes, both in the acquired cholesteatoma and in the skin of bony portion of the external auditory canal near fibrocartilaginous annulus in more serious clinical picture of cholesteatoma than in less serious clinical picture of cholesteatoma ($p < 0.01$). There was statistically significant difference of expression of PCNA, Ki-67, COX-2, CD 4 and CD 8 lymphocytes between all cholesteatoma samples and the skin of bony portion of the external auditory canal ($p < 0.05$) and statistically significant difference of expression of those markers between the skin of bony portion of the external auditory canal and retroauricular skin ($p < 0.05$). **Conclusion.** Inflammation of the skin of bony portion of the external auditory canal is a milestone in pathogenesis of acquired middle ear cholesteatoma. Expression of CD 4 lymphocytes can be the prognostic factor for acquired cholesteatoma clinical picture development. We found so much diversity in biological behavior through very different levels of cholesteatoma development. Expression of Ki-67 in acquired middle ear cholesteatoma is a reliable and stable marker of proliferation for acquired middle ear cholesteatoma.

Key words:
cholesteatoma; histology; immunohistochemistry; biological markers.

Apstrakt

Uvod/Cilj. Konstantna proliferacija i periodične infekcije su glavne kliničke karakteristike stečenog holesteatoma srednjeg uva. Cilj ove studije bio je da se istraže imunohistohemijske karakteristike kože i holesteatomskog procesa u obližnjem tkivu i tako prostudira etiologija i razvoj stečenog holesteatoma srednjeg uva. **Metode.** Istraživali smo kliničke, histološke i imunohistohemijske karakteristike holesteatoma 50 operisanih bolesnika sa stečenim holesteatomom srednjeg uva. Klasifikovali smo sve uzorke prema kliničkim karakteristikama holesteatoma kao što su de-

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strukcija kosti, prisustvo infekcije ili proširenost holesteatomskog procesa i histološkim karakteristikama holesteatoma kao što su keratinizacija, inflamatorni proces i ekstraćelijska proliferacija matriksa. Koristili smo mišija monoklonska antitela za proliferišući ćelijski nuklearni antigen (MAbs za PCNA), Ki-67, COX-2, CD 4 i CD 8 limfocite da bi istražili ekspresiju ovih karakteristika u holesteatomu i u kontrolnom tkivu kože. Statističke analize izvedene su korišćenjem SPSS za Windows, verzija 16.0 (SPSS, Chicago, IL, USA). Koristili smo *t*-test za nezavisne grupe, Spirmanovu analizu korelacije i Mann-Whitney U test za statističku analizu. **Rezultati.** Ekspresija PCNA, Ki-67, COX-2 i CD 8 limfocita u holesteatomima sa težom kliničkom slikom bila je podjednaka kao u holesteatomima sa lakšom kliničkom slikom. Postojala je statistički značajno viša koncentracija CD4 limfocita u uzorcima stečenog holesteatoma i u uzorcima kože koštanog dela spoljnog slušnog hodnika, u blizini fibrokartilaginoznog anulusa, u holestetomima sa težom kliničkom slikom nego u holesteatomima sa lakšom kliničkom slikom ($p < 0,05$). Postojala je statistički značajna razlika ekspresije PCNA, Ki-67, COX-2, CD-4 i CD 8 limfocita izmedu uzorka holesteatoma i uzoraka kože koštanog dela spoljnog slušnog kanala svih operisanih bolesnika ($p < 0,05$) i statistički značajna razlika u ekspresiji PCNA, Ki-67, COX-2, CD-4 i CD 8 limfocita u koži koštanog dela spoljnog slušnog hodnika u odnosu na retroaurikularnu kožu ($p < 0,05$). **Zaključak.** Inflamacija kože koštanog dela spoljnog slušnog hodnika je prekretnica u patogenezi stečenog holesteatoma srednjeg uva. Ekspresija CD4 limfocita može biti prognostički faktor u razvoju kliničke slike stečenog holesteatoma. Našli smo toliko različitosti u biološkom ponašanju holesteatoma na različitim nivoima kliničkog razvoja. Ekspresija Ki-67 u stečenom holesteatomu srednjeg uva je pouzdan i stabilan marker proliferacije stečenog holesteatoma.

Ključne reči:
holesteatom; histologija; imunohistohemija; biološki pokazatelji.

Introduction

Cholesteatoma represents cystic, benign, pseudotumor lump (sac), limited with keratinized squamous epithelium with stroma of granulation tissue, which occurs in various places in body, including within the middle ear. In 1964 Gray [1] described cholesteatoma as "skin in the wrong place", without hair and glands, while Sade [2] defined cholesteatoma as "the existence of the squamous epithelium of the tympanic cavity, which produces macroscopic amounts of keratin". Cholesteatoma is a chronic disease of the middle ear which resorbs bone [2]. Cholesteatoma can damage hearing and vestibular function and sometimes leads to exocranial and endocranial life-threatening complications. Not a single theory has been able to explain the clinical characteristics of acquired cholesteatoma: uncoordinated proliferation, invasion, migration, altered differentiation, aggressiveness and recidivism [3]. Permanent proliferation and periodical infection are the main clinical characteristics of acquired middle ear cholesteatoma. The aim of this study was to research immunohistochemical characteristics of the skin along with cholesteatoma processes in the nearby tissue significant for etiology and development of acquired middle ear cholesteatoma.

By expression of cytokeratin, cytokines and proliferation markers such as proliferating cell nuclear antigen (PCNA) and Ki-67, epithelial proliferation of the cholesteatoma may be explored. PCNA is used to analyze the synthesis of deoxyribonucleic acid (DNA) during the S phase of the cell cycle, which is related to DNA replication, repair, and cellular apoptosis. PCNA is therefore a non-specific indicator of proliferation of the epithelium. PCNA expression is increased in chronic inflammatory processes. Thus PCNA is a marker suitable for the proliferation assay of cholesteatoma matrix, where the expression of PCNA is higher than in the epidermis. Ki-67 nuclear antigen is a marker of cell proliferation which is expressed in all active phases of the cell cycle (G1, S, G2 and M), while it is not expressed in the inactive phase of the cell cycle (G0). It is assumed that Ki-67 is responsible for chromosome coupling and decongestion, chromosome stabilization and protection and regulation of the symmetric distribution of the nuclear proteins in cell division [4]. Depending on the presence of Ki-67 in the nucleus, in the nucleolus or in the nucleoplasm, expression of the Ki-67 is determined as a granular, diffuse or mixed cell type [5]. Ki-67 has a specific immunoreactivity: it is detectable in the basal and suprabasal layers of the acquired cholesteatoma matrix, while Ki-67 appears only in the basal layer of the epithelium of normal skin [6]. Ki-67 and PCNA are particularly concentrated in the skin near tympanic annulus, where it takes the clip skin of external auditory canal suitable for histopathologic and immunohistochemical analyses. COX-2 (cyclooxygenase 2) is an enzyme generated by decoding PTGS2 (prostaglandin-endoperoxydsynthetize 2) gene. COX-2 is involved in conversion of arachidonic acid to prostaglandin H2 – the precursor of prostacyclin and thromboxane A2, the mediators of the inflammatory process [7]. COX-2 is inactive in healthy cells. The activation of COX-2 occurs in proliferating cells during the development of inflammatory or tumor processes in the body. COX-2 occurs as a response to extracellular stimulation, proinflammatory cytokines or cell growth factors [8, 9].

By expression of immune cells markers, such as CD4 and CD8 T cells in the cholesteatoma, as well as Th1 and Th2 helper immune cells, immune process in the cholesteatoma development can be followed. CD4 and CD8 T lymphocytes are the effector cells which act as cytotoxic or immune cells in the humoral immune response in the cholesteatoma development, after their maturation in the lymph nodes and their migration to the site of infection [10].

Methods

The research was designed as cross-sectional, study. The study included 50 patients between 10–77 years of age,

both sexes (34 male, 16 female) who were operated with the diagnosis of acquired middle ear cholesteatoma. The study was conducted from 2012–2015 at the Ear, Nose and Throat (ENT) Clinic, Military Medical Academy (MMA) in Belgrade and the Institute of Pathology, MMA in Belgrade, according to the provisions of the Declaration of Helsinki. Subject got the approval from the Ethics Committee of Military Medical Academy on April 1, 2012 according to the order of the Head of the Academy (classified) 3232-1. The main criterion for inclusion of the patients in the study was the diagnosis of acquired middle ear cholesteatoma (Figure 1).

Criteria for the exclusion from the study were associated diseases with the acquired middle ear cholesteatoma, such as malignant neoplasm whichever origin it has and chronic diseases of the skin of the external auditory canal and / or auricular regions as eczema, psoriasis, lupus, etc.

We performed tympanoplasty in all pediatric patients (range 10–19 years old) and in most adults (42 patients score). We performed radical tympanomastoidectomy in 8 adults, reoperations of recurrent cholesteatoma in 6 and operations in 2, because of diffuse cholesteatoma (Table 1). We used high resolution temporal bone computed tomography (CT) to establish the diagnosis of recurrent cholesteatoma before reoperation. Nowadays, improvements in magnetic resonance imaging (MRI) techniques led to getting more accurate details of the cholesteatoma using delayed contrast enhanced T1 weighted imaging and diffusion-weighted imaging [11].

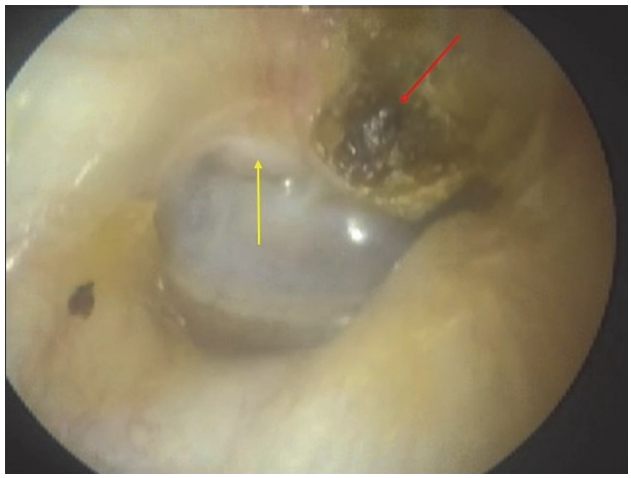

Fig. 1 – Otomicroscopic view of attic cholesteatoma. Cholesteatoma crust (red arrow). Spatium Von Troeltsch - filled with spread attic cholesteatoma (yellow arrow).

Table 1

Distribution of patients according to cholesteatoma clinical classification and type of surgery

Acquired cholesteatoma localization	Number of patients	Tympanoplasty	Radical tympanoplasty
Attic cholesteatoma	21	21	0
Pars tensa cholesteatoma	17	17	0
Diffuse cholesteatoma	3	1	2
Recidivism	9	3	6
Number of operations	50	42	8

The cases were categorized according to the degree of bone destruction (29 patients with more and 21 patients with less bone destruction), the presence of infection (36 patients with and 14 patients without infection) and cholesteatoma extension (34 patients with more and 16 patients with less cholesteatoma extension). We also investigated the correlation of the expression of PCNA, Ki-67, COX-2, CD 4 and CD 8 lymphocytes and the histological characteristics of the cholesteatoma to determine the possible presence of any differences in expression of proliferation and inflammation cell markers according to the degree of keratinization, inflammation or extracellular matrix proliferation. This study also looked at correlation between the immune histochemical and the clinical characteristics of cholesteatoma [12]. Cases were categorized according to clinical findings, i.e. having more bone destruction, cholesteatoma induced erosion of 2 or 3 auditory ossicles, leading to ossicular discontinuity along with erosion of the wall of the middle ear or having less bone destruction with no ossicle damage or one auditory ossicle damage because of the cholesteatoma process. A positive microbial swab before or during surgery indicated the presence of bacterial infection in the cholesteatoma while a negative swab indicated absence of infection. In the group categorized as having more cholesteatoma extension, cholesteatoma was found in all three compartments of the middle ear (i.e. epitympanum, antrum and mastoid cells); in contrast, cholesteatoma extension in one or two compartments of the middle ear defined cases with less cholesteatoma extension (i.e. epitympanum and protympanum).

Samples of cholesteatoma from all 50 cases were soaked in formalin, embedded in paraffin and cut into 4 mm sections using a microtome. All samples were taken from tissue representing complete resection of the cholesteatoma. The slides were deparaffinized, hydrated and washed with TRIS-buffered saline plus 0.5% Tween 20 (TBST buffer). Samples were then subjected to microwave treatment for 30 min in citrate buffer (pH = 6.0) to retrieve the antigens. Endogenous peroxidase activity was blocked using water plus 3% H_20_2 for 30 min. Finally, the cells were characterized using mouse monoclonal antibodies that reacted with the following human proteins: PCNA, Ki-67, COX-2 and CD8 (Serotec); CD4 (Novocastra). Diaminobenzidine was applied for 10 min as a chromogen. The slides were counterstained with Mayer's hematoxylin, washed in water, dehydrated in increasing concentrations of ethanol, cleared in xylene and put in Canada balsam [13, 14].

The number of immune cells that showed positive staining with the cell-specific antibodies was reported semi-quantitatively. Expressions of PCNA, Ki-67, COX-2, CD 4 and CD 8 lymphocytes were scored as follows: 0 – no staning; 1 – 25% positive staining; 2 – 25–50% positive staining; 3 – 50–75% positive staining; 4 – 75–100% positive staining, in one high-powered microscope field (HPF). The histological characteristics of cholesteatomas were scored using a semi-quantitative method to obtain "indexes" for the characteristics. Keratinization was scored as follows: 0 – no keratinization; 1 – low level of keratinization; 2 – moderate level of keratinization; 3 – high level of keratinization. Inflammation/inflammatory cell infiltration was scored as follows: 0 – no inflammatory cell infiltration; 1 – low level of inflammatory cell infiltration i.e. fewer than 5 cells in one HPF; 2 – moderate level of inflammatory cell infiltration, i.e. 5–20 cells in one HPF; 3 – high level of inflammatory cell infiltration, i.e. more than 20 cells in one HPF. Extracellular matrix proliferation (collagen) was scored as follows: 0 – no collagen; 1 – low level of collagen expression; 2 – moderate level of collagen expression; 3 – high level of collagen expression [15, 16].

We used *t*-test for independent groups and the Mann-Whitney U test to statistically analyze the results, obtained. Spearman's correlation analysis was used to test the correlation between two cholesteatoma characteristics. A p value less than 0.05 was considered to be statistically significant in all statistic analyses, while a p value less than 0.01 was considered to be highly statistically significant.

Results

The mean estimated score of keratinization for all samples was 2.20 ± 0.904, that is a moderate level of keratinization. More than 10 layers of keratinocytes in the cholesteatoma matrix were followed by expressed inflammatory cells infiltration into subepithelial layer of cholesteatoma perimatrix. The mean estimated index of inflammation/inflammatory cell infiltration for all samples was 2.16 ± 1.017, that is a moderate level of inflammation. The perimatrix was dominantly infiltrated by mononuclear cells with diffuse or aggregate distribution. The degree of extracellular matrix proliferation was very different in cholesteatoma perimatrix. The mean estimated index of extracellular matrix proliferation for all samples was 1.92 ± 1.158, that is a moderate level of collagen expression.

We studied and compared the expression of cellular proliferative and inflammatory markers PCNA, Ki-67, COX-2, CD4 and CD8 T cells in 3 tissue preparations of each of the operated patients: a composition of cholesteatoma, a composition of skin of the external auditory canal and preparation of the retroauricular skin regions of the total of 50 treated patients with the acquired middle ear cholesteatoma. Also, we investigated the effect of proliferative and inflammatory parameters in the development of acquired cholesteatoma, comparing the expression of PCNA, Ki-67, COX-2, CD4 and CD8 T lymphocytes in different kinds of cholesteatoma grouped according to histological and clinical characteristics of the acquired cholesteatoma.

PCNA was strongly detectable in cholesteatoma, in relation to the skin of the external auditory canal and to the skin of the retroauricular regions. The expression of PCNA was detected in proliferating cells of the basal layer and suprabasal layer of the cholesteatoma matrix and rarely in the upper layers of the matrix. PCNA expression was detected in the granular layer of the matrix when the proliferation of keratinocytes in the matrix of acquired cholesteatoma was increased, which supports the theory that PCNA is a non-selective marker of cell proliferation. PCNA expression was less in acquired cholesteatoma without infection, but with higher keratin deposits and with increased keratinization. Compared to acquired cholesteatoma, PCNA expression in the skin of the external auditory canal and in the skin of the retroauricular region is less frequently detected, especially in the skin of the retroauricular region, where PCNA staining was mainly detected in individual cells.

Ki-67 showed a great detectability in cholesteatoma, in relation to the skin of the external auditory canal and of the retroauricular region. The increased number of proliferating keratinocytes, which have a specific Ki-67 immunoreactivity, was detected in the basal and suprabasal layer, and less frequently in the upper layers of the matrix of all examined cholesteatoma. Expression of keratinocyte marker Ki-67 was present in all active phases of the cell cycle, which was best illustrated by a different type of nuclear staining (Figure 2).

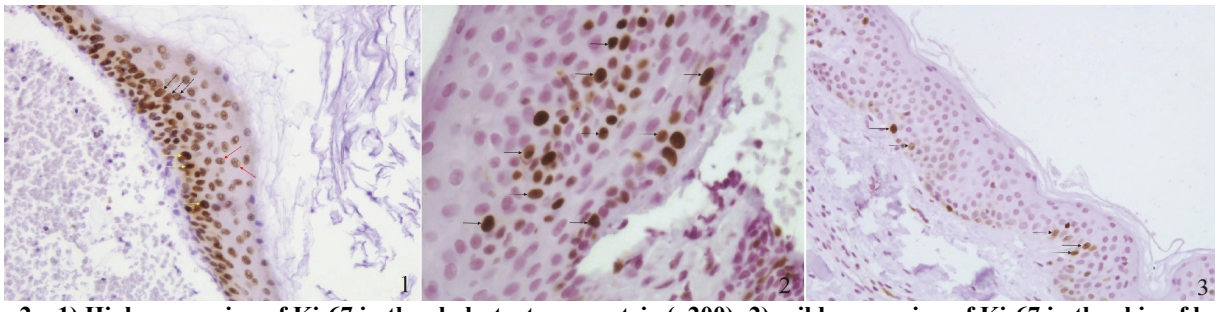

Fig. 2 – 1) High expression of Ki-67 in the cholesteatoma matrix (x200); 2) mild expression of Ki-67 in the skin of bony portion of the external auditory canal (x400); 3) low expression of Ki-67 in the retroauricular skin (x200). Different Ki-67 nuclear staining in keratinocytes: granular type (red arrows), diffuse type (yellow arrows) and mixed type (blue arrows).

COX-2 was detected in all samples of acquired cholesteatoma, mainly as a low to moderate staining expressed (immunoreactivity 50%) of the matrix of cholesteatoma. The estimated expression of COX-2 in the acquired cholesteatoma (immunoreactivity over 50%) was in a small number of cases (18% of total respondents), and followed by prominent lymphocyte proliferation in perimatrix, and increased keratinocytes proliferation in the matrix. Also there was a significant diversity in the expression of COX-2 in the samples of the skin of external auditory canal. COX-2 was almost undetected in the samples of the retroauricular skin.

CD4 marker was perfectly manifested in the subpopulation of helper T lymphocytes in the samples of acquired cholesteatoma. Abundant diffuse infiltrates of CD4 lymphocytes were detected in the perimatrix or there were smaller or larger deposits of cells in the so-called cluster formations. The expression of CD4 cells in the matrix of the cholesteatoma was generally scarce to poorly expressed, while CD4+ cells morphology was diverse, predominantly rounded, occasionally atypical, elongated, or star shaped, with more or less nucleus hyperhromasia.

The expression of CD4 lymphocytes was mostly poor to moderately expressed in the samples of skin of the external auditory canal with a predominance of subepithelial CD4+ cells distribution, while detectability of CD4+ cells in the retroauricular skin was feeble (Figure 3).

The expression of CD8 lymphocytes was lower than the expression of CD4 in the samples of the acquired cholesteatoma with subepithelial cytotoxic T lymphocytes distribution in the cholesteatoma perimatrix. The expression of CD8 lymphocytes in the skin of the external auditory canal was less than the expression of CD8 lymphocytes in acquired cholesteatoma, while expression of CD8 lymphocytes in the retroauricular skin was minimal.

Results of comparing tissue samples of acquired cholestaetoma and skin of the external auditory canal and retroauricular skin according to the clinical, histopathological and immunohistochemical characteristics of acquired cholesteatoma are presented in Tables 2 and 3. There was not astatistically significant difference in index correlation of keratinization, inflammatory infiltration and proliferation of collagen according to the degree of bone destruction of the acquired cholesteatoma.

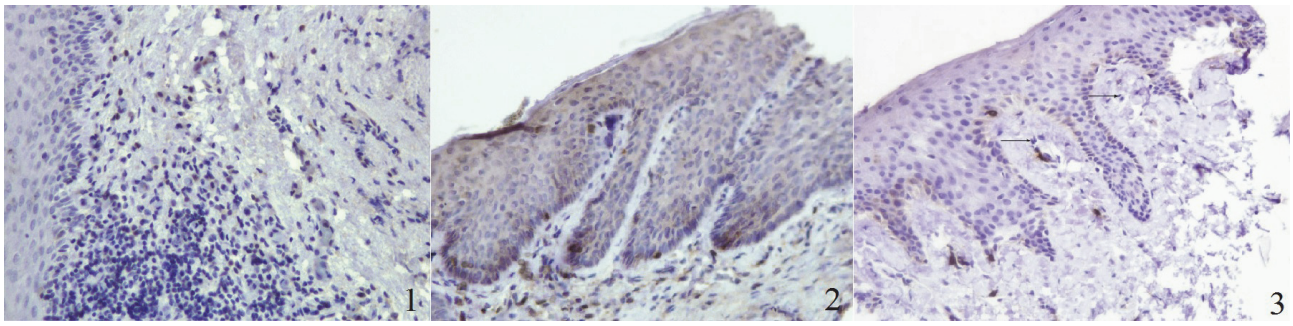

Fig. 3 – 1) Perimatrix cluster infiltration of the acquired cholesteatoma with CD4 lymphocytes and individual CD4 + cells expression in the matrix (x 200); 2) Subepithelial distribution of T lymphocytes expressed with CD4 marker in the skin of the external auditory canal (x200); 3) Individual T lymphocytes (black arrows) expressed with CD4 marker in the retro auricular skin (x200).

Table 2

Histological and immunohistochemical characteristics of cholesteatoma according to degree of bone destruction of the acquired middle ear cholesteatoma

Bone destruction	Keratinization	Inflamm. infiltrate	Collagen	PCNA	Ki-67	COX2	CD4	CD8
More bone destruction	29	29	29	29	29	29	29	29
Index	2.10	2.28	1.97	3.79	3.41	2.39	3.93	3.07
SD	0.976	0.960	1.149	0.412	0.780	0.620	0.408	0.913
Less bone destruction	21	21	21	21	21	21	21	21
Index	2.33	2.00	1.86	3.81	3.00	2.03	3.24	2.45
SD	0.796	1.095	1.195	0.402	0.949	1.076	0.982	1.061
χ^2 test	2.149	2.160	0.144	0.021	3.210	2.278	25.384	1.149
Df	3	3	3	1	3	2	1	2
p	0.542	0.540	0.986	0.886	0.360	0.320	0.001*	0.563

SD – standard deviation; Df – degree of freedom; *p* – probability; *highly statistically significant; PCNA – proliferating cell nuclear antigen; COX2 – cyclooxigenase 2.

Table 3

The expression of cell proliferation and inflammation markers in the skin of external auditory canal and retroauricular skin according to degree of bone destruction of the middle ear cholesteatoma

Bone destruction	PCNA		Ki-67		COX2		CD4		CD8	
	ssh	ra	ssh	ra	ssh	ra	ssh	ra	ssh	ra
More bone destruction	29	29	29	29	29	29	29	29	29	29
Index	2.62	1.69	2.24	1.55	2.32	1.69	3.21	1.71	2.12	1.33
SD	0.622	0.471	0.830	0.506	0.871	0.471	0.904	0.609	0.734	0.453
Less bone destruction	21	21	21	21	21	21	21	21	21	21
Index	2.71	1.71	2.05	1.43	2.12	1.76	2.24	1.66	1.96	1.23
SD	0.463	0.463	0.498	0.507	0.740	0.539	0.692	0.512	0.634	0.412
χ^2 test	1.551	0.035	3.658	0.739	3.353	1.414	18.443	1.409	1.149	1.763
Df	2	1	3	1	2	2	3	1	2	1
p	0.460	0.851	0.301	0.390	0.187	0.493	0.001*	0.235	0.563	0.184

SD – standard deviation; Df – degree of freedom; *p* – probability; *highly statistically significant; ssh – external auditory canal; ra – retroauricular skin; PCNA – proliferating cell nuclear antigen; COX2 – cyclooxygenase 2.

There was a statistically significant difference in the index of keratinization according to the presence/absence of cholesteatoma infection ($p = 0.028$), showing that keratinization was more expressed in cholesteatoma without infection. There was a statistically significant difference in the index of inflammatory infiltration of cholesteatoma ($p = 0.049$) according to absent/present cholesteatoma infection, whereby cholesteatoma with infection tended to show mainly conspicuously marked inflammatory cell infiltration (24 of 36 respondents, or 67% of the patients in the group of cholesteatoma with infection had markedly pronounced inflammatory infiltrate) in the histopathological examination (Figure 4).

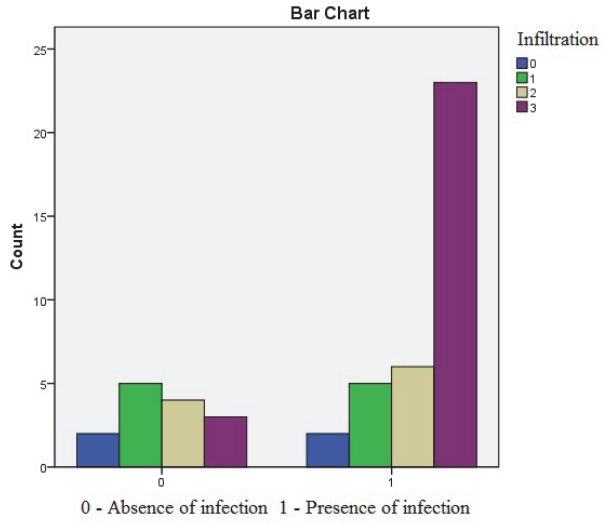

Fig. 4 – Inflammatory infiltrate index in the group of patients with acquired cholesteatoma of the middle ear with absent/ present infection.

0 – no inflammatory cell infiltration; 1 – low level of inflamatory cell infiltration; 2 – moderate level of inflammatory cell infiltration; 3 – high level of inflammatory cell infiltration.

There was no statistically significant difference in the index of histopathologic features of acquired cholesteatoma and the expression of markers PCNA, Ki-67, COX2 and CD8 lymphocytes in cholesteatoma between the groups of patients with more bone destruction and the group of patients with less bone destruction in the middle ear. There was a highly statistically significant difference in the expression of CD4 lymphocytes in the cholesteatoma ($p = 0.001$) and in the expression of CD4 lymphocytes in the skin of the external auditory canal ($p = 0.001$) compared to the level of bone destruction (Tables 2 and 3).

There was a statistically significant difference in the expression of CD4 lymphocytes in cholesteatoma ($p = 0.041$) and in the skin of the external auditory canal ($p = 0.048$) according to the presence/absence of the infection. There was a statistically significant difference in the expression of CD4 lymphocytes in cholesteatoma ($p = 0.007$) and in the expression of CD4 lymphocytes in the skin of the external auditory canal ($p = 0.020$) according to the extensiveness of cholesteatoma. There was a strong CD4 + cell staining in the acquired cholesteatoma and in the skin of the external auditory canal in the cholesteatoma samples with higher extensiveness of the cholesteatoma process.

We used Spearman's correlation coefficient for testing the relationship of the histopathological features in the acquired middle ear cholesteatoma. For histopathological characteristics of cholesteatoma it showed highly statistically significant negative correlation between keretanization and inflammatory infiltration in cholesteatoma ($p = 0.008$), highly statistically significant negative correlation between keratinization and collagen proliferation in cholesteatoma ($p = 0.002$), and a highly statistically significant positive correlation between inflammatory infiltration and collagen proliferation in cholesteatoma ($p = 0.001$). Keratinization in the acquired middle ear cholesteatoma was particularly pronounced in lower inflammatory infiltration and less collagen proliferation. Inflammatory infiltration in the middle ear acquired cholesteatoma was correlated with the collagen development in the perimetrix.

Expression of the markers PCNA, Ki-67, COX-2, CD4 and CD8 T lymphocytes in cholesteatoma according to histopathological characteristics of cholesteatoma showed equal values, with no statistically significant differences.

There was a highly statistically significant difference in expression of all markers PCNA, Ki-67, COX-2, CD4 and

CD8 T lymphocytes in cholesteatoma compared to the expression of the same markers in the skin of the external auditory canal and retroauricular skin. Also the expression of all markers PCNA, Ki-67, COX-2, CD4 and CD8 T lymphocytes in the skin of the external auditory canal was highly statistically different from the expression in the retroauricular skin.

Discussion

Examining the 50 operated patients with acquired middle ear cholesteatoma in our study, we obtained statistically significant higher expression of CD4 T lymphocytes in acquired cholesteatoma with more severe clinical picture, greater bone destruction, present infection as well as increasing extensiveness of the cholesteatoma process. We got a similar result for the expression of CD4 T lymphocytes in the skin of the external auditory canal. CD8 T cells did not show such a distribution in the acquired cholesteatoma and in the skin of the external auditory canal as CD4 T lymphocytes. CD8 T lymphocytes expressed less than CD4 T lymphocytes in cholesteatoma and in surrounding skin. Possibly, CD 8 T lymphocytes cytotoxicity had less importance in the immunological processes in the acquired cholesteatoma. CD 8 T cells had equal distribution and equal activity in different clinical groups of cholesteatoma. The other authors also favored the humoral immune response in the development of acquired cholesteatoma, which was closely linked to the presence of Langerhans cells in acquired cholesteatoma and the increased presence of CD4 T lymphocytes [17, 18]. CD4 T lymphocytes were the main carriers of the inflammatory infiltrate in our study, especially in strikingly expressed inflammatory infiltrates which could be found in 67% of respondents with infection. Lymphocytic infiltrates were predominantly stained in the subepithelial perimatrix in diffuse distribution with the possibility of good communication to the cholesteatoma matrix. Inflammatory infiltration was statistically significantly higher in acquired cholesteatoma with infection in comparison with acquired cholesteatoma without infection, which was evidenced in some earlier articles [15]. Ma et al. [19] showed, by comparing different places for cholesteatoma sampling, that the clinical picture of acquired cholesteatoma went with large inflammatory infiltrate in permatrix. Cholesteatoma cases sampled from purulent, bone destruction parts of the middle ear had the most expressed inflammation [19]. Acquired cholesteatoma developing for years got the aggressive, bone destruction form during the direct contact between cholesteatoma and bones. Immunological events in the inflammatory infiltrate of cholesteatoma perimatrix were responsible for bone destruction of the middle ear. Inflammatory infiltrate of the acquired middle ear cholesteatoma was correlated with the development of collagen in perimetrix in our study. Collagen was produced by fibroblasts in acquired cholesteatoma perimatrix as sequelae in the final stages of the inflammatory process [13]. Keratinisation of the acquired middle ear cholesteatoma was less pronounced due to increased inflammatory infiltrate, increased extracellular matrix, or collagen in our study. More expressed inflammatory process in cholesteatoma perimatrix led to more bone destruction in the middle ear. The phenotypic diversity of cholesteatoma may explain lesser or greater inflammation, but not its origin [19].

Keratinization was negatively correlated with the infection of acquired cholesteatoma. Keratinization was statistically significantly higher in cholesteatoma without infection in comparison with cholesteatoma with infection in our study. Regular bulbous appearance of adherent keratin deposits in the cholesteatoma without infection, with a thin matrix of cholesteatoma is a typical histopathological picture of the slowly growing acquired cholesteatoma without exacerbation of infection. Thin layers of the cholesteatoma matrix with greater keratinization suggest the problem of keratinocyte differentiation in the cholesteatoma development. More keratin accumulation in keratinocytes during corneocytes creation contibutes to a process of separating the lamellar layers and the keratin creating bulb keratin as a standard phenotypic characteristics of these cholesteatoma. The apoptosis of corneocytes which occurs not only in the horny layer of the matrix of the cholesteatoma (at 2–4 splitter), but also in the lower, contibutes to a great amount of amorphous keratin. According to our research, keratinization correlated with abnormal keratinocyte differentiation. There was a poorly expressed inflammatory infiltration in perimatrix of these cholesteatomas, which probably means that there is a poor and paracrine communication between the matrix and the perimatrix. The reason for the accelerated maturation and disturbed differentiation of keratinocytes accompanied by apoptosis of epithelial cells should be seek searched in the intercellular matrix developments or physical factors as a type of pressure, acid-base status, hypoxia, etc. [20]. The expression of cell proliferation markers PCNA and Ki-67 in keratinocytes in our study did not show statistically significant differences according to the clinical and histopathological characteristics of the acquired cholesteatoma. The test results obtained from Ki-67 expression of keratinocyte proliferation in the acquired cholesteatoma in children and adults are controversial. Welkoborsky [9], and Dornelles et al. [13] showed no difference in keratinocyte proliferation obtained from the children and from the adult form of cholesteatoma, while Aslıer et al. [14] showed a statistically greater Ki-67 expression in adults cholesteatoma compared to children cholesteatoma. Li et al. [16] showed significantly higher Ki-67 expression in cholesteatoma with pronounced bone erosion than in cholesteatoma with less bone erosion. Sanli et al. [21] showed no significant differences in Ki-67 expression in primary and recurrent acquired cholesteatoma. The proliferation of keratinocytes was significantly higher in the skin of the external auditory canal than in the retroauricular skin, but also the proliferation of keratinocytes was significantly higher in cholesteatoma matrix than in the skin of the external auditory canal, analyzing the expression of PCNA and Ki-67, which proved to be expected and was in line with the findings of other authors [6, 22, 23].

Clinical verification of acquired cholesteatoma infection was obtained by positive bacteriological swab test. In our study 72% cholesteatoma samples were infected by indi-

vidual or mixed bacterial flora. The infectious agent obtained initiation of cholesteatoma matrix causing additional activation of the keratinocytes proliferation. The infectious agent can be proliferation of the endotoxin from the bacterial cells wall, fragments of the keratin debris, biofilm, etc. Recent studies show a significant role of bacterial biofilms which is identified as the infectious agent and trigger in the development of chronic suppurative otitis with cholesteatoma [24]. The presence of bacterial biofilms from the accumulated keratin may be the evidence of latent cholesteatoma infection in the group of 28% of the patients with ear swab – negative tests, in our study. Bacteria or fungi inside the biofilms were resistant to antibiotics and the immune response of the host and led to a state of chronically infected cholestetaoma [25].

By exploring histopathological features of acquired cholesteatoma it is shown that there is much diversity of cholesteatoma structure, which correlates with the stage of cholesteatoma development. The heterogeneous morphological images of the acquired cholesteatoma do not show precise diversity of the biological behavior of cholesteatoma in different samples. The histopathological findings of the acquired cholesteatoma reflect only one moment in the cholestatoma development. The results of the histological examination should therefore be commented with consideration of the time when the sample tissue was taken and anatomical site from which the sample tissue was taken.

Conclusion

We found much diversity in biological behavior through very different levels of cholesteatoma development. Keratinisation index of acquired middle ear cholesteatoma was higher in the absence of infection. Inflammatory cell infiltrate index of acquired middle ear cholesteatoma was higher in cases with infection. Keratinisation index had negative correlation with inlammatory cell infiltrate index and extracellular matrix proliferation index of acquired middle ear cholesteatoma. Inflammatory infiltrate had positive correlation with colagen development in perimatrix of acquired middle ear cholesteatoma. Inflammation in the skin of bony portion of the external auditory canal is a milestone in etiology and pathogenesis of acquired middle ear cholesteatoma. Expression of Ki-67 in acquired middle ear cholesteatoma is a reliable and stable marker of proliferation for acquired middle ear cholesteatoma. Expression of CD 4 lymphocytes can be a prognostic factor for acquired cholesteatoma clinical picture development.

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