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From Cell Biology to Tissue Engineering

Diagnostic value of matrix metalloproteinase 9 and tissue inhibitor of matrix metalloproteinases 1 in cholesteatoma

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Summary. Objectives: Matrix metalloproteinase 9 (MMP-9), able to degrade type IV collagen, plays a key role in inflammatory cell migration as well as in the destructive behaviour of cholesteatoma. The aim of our study was to compare the expression of MMP-9 and TIMP-1 in cholesteatoma tissue and in the concentrations in serum and plasma concentrations.

Material and Methods: Twenty five adult patients suffering from cholesteatoma (a study group) were included in the study. A comparison group consisted of 25 adult patients admitted to hospital due to nasal septum deviation. MM-9 and TIMP-1 serum and plasma concentrations as well as proteins' expressions in cholesteatoma tissues (study group) and normal retroauricular skin specimens (control group) were evaluated. MMP-9 and TIMP-1 concentrations were measured using enzyme-linked immunosorbent assay (ELISA). Cholesteatoma tissues and normal retro-auricular skin specimens were evaluated immunohisto-chemically.

Results: In the study and comparison groups, MMP-9 and TIMP-1 concentrations were similar with no significant difference within the groups. In cholesteatoma tissues, the expression of the investigated enzyme and its inhibitor was higher than in normal skin specimens, limited mostly to cholesteatoma perimatrix.

Conclusion: Cholesteatoma may be limited to the middle ear or parts of the temporal bones. Our findings suggest better clinical usefulness of MMP-9 and TIMP-1 expression in cholesteatoma tissues than either serum or plasma levels of these proteins. It might suggest that the higher the expression of MMP-9 the stronger the inflammation -accompanied cholesteatoma.

Key words: Cholesteatoma, Matrix metalloproteinase 9, Tissue inhibitor of metalloproteinase, Serum, Plasma

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Introduction

Cholesteatoma of the middle ear may lead to the destruction of middle and inner ear structures, causing hearing loss, vestibular dysfunction and facial paralysis, as well as lethal intracranial complications. The discussion on cholesteatoma etiology, epidemiology, pathogenesis and surgery is still open. It has been proved that some groups of enzymes, such as cathepsins, and lysosomal exoglycosideases, play a role in the destructive behaviour of cholesteatoma. The increase in these enzymes' activities has been observed in several inflammatory diseases. Inflammation is always observed in the microenvironment of cholesteatoma and a catabolic process associated with their inflammatory condition, such as cholesteatoma, seems to be crucial in the pathogenesis of that potentially life-threatening disease. The enzymes that are capable of degrading extracellular matrix components belong to matrix metalloproteinases.

Matrix metalloproteinases (MMPs) are a family of

structurally related zinc-dependent endopeptidases, including collagenases, gelatinases (matrix metalloproteinase 9 and 2), stromelysins and matrylysins. All MMPs are synthesized as pre- and pro-enzymes and secreted as inactive pro-MMPs in most cases. Zymogens are activated by disruptions of the Cys-Zn^{+} interaction. MMPs are capable of degrading the basement membrane and all components of extracellular matrix (ECM). MMPs play an important role in many normal biological processes such as bone remodeling, wound healing, angiogenesis, apoptosis, embryonic development, blastocyst implantation and organ morphogenesis. MMPs also participate in pathological processes (e.g. cardiovascular disease, arthritis, neurological disease, Alzheimer's disease, periodontal disease and cancer) (Nagase and Woessner, 1999). Matrix metalloproteinase 9 (MMP-9) is also capable of degrading type IV collagen, which is the main component of the basement membrane and leads to cell migration, angiogenesis, growth of the tumor and metastasis (Vihinen and Kahari, 2002; Murray et al., 2004). It was widely proved that keratinocyte migration and proliferation as well as angiogenesis played an important role in the development of congenital and acquired, primary and secondary cholesteatoma (Olszewska et al., 2004). As soon as the cholesteatoma epithelium begins to hyperproliferate, the destructive behaviour of cholesteatoma is triggered. Hyperproliferative activity of cholesteatoma keratinocytes was evidenced by the expression of cytokeratines, Ki-67 and PCNA (Proliferating Cell Nuclear Antigen). Angionesesis in cholesteatoma is induced in two ways: first by monocytes, macrophages and leucocytes present within cholesteatoma perimatrix, which are capable of producing angiogenic factors, such as fibroblast growth factor, vascular endothelial growth factor, transforming growth factor alpha. On the other hand, keratinocytes themselves stimulate the release of angiogenic factors. Enhanced vascularization in cholesteatoma may also cause a continuous and pathologic growth of cholesteatoma mass. Up to 811 genes were identified as up-regulated in cholesteatoma compared to healthy skin. It was proved that MMP-1, MMP-9, MMP-10 and MMP-12 were up-regulated in cholesteatoma. The strong expression of MMP-9 and its substrate osteopontin 9 has been demonstrated in a group of patients with cholesteatomas but the authors found some cases with a low expression of that protein with a high expression of S100A and GJB2 genes, which suggests a similar pattern of skin and cholesteatoma as well as a similar expression of differentiation and apoptosis-related genes as does normal epithelium (Klenke et al., 2012).

Tissue Inhibitors of Metalloproteinases (TIMPs) are the major endogenous regulators of MMP activities, and four homologous TIMPs have been identified to date. TIMP-1 binds and inhibits MMP-9 by forming noncovalent 1:1 stoechiometric complexes. It was observed that increased expression of TIMP-1 correlated with overexpression of MMP-9. TIMP-1 induced the growth of normal cells including keratinocytes, chondrocytes, fibroblasts, epithelial and endothelial cells. In pathological conditions, overexpression of TIMP-1 was observed in hepatoma, breast carcinoma and osteosarcoma (Lambert et al., 2004). TIMPs may stimulate tumour growth and inhibit cell apoptosis (Guedez et al., 1998; Hewitt et al., 2000). Therefore, it was suggested that TIMP-1 might be considered as the factor to play a dual role in the regulation of tumour progression. An increased rate of dead cells formed during the differentiation of keratinocytes may likely be related to apoptosis observed in cholesteatoma (Olszewska et al., 2006, 2013).

The correlation between serum MMPs and their tissue inhibitors' activities with their expressions in cholesteatoma tissue was only marginally considered in that disease. There are no data concerning the activity of MMP-9 and TIMP-1 in sera obtained from the blood of cholesteatoma patients. Lack of interest is surprising in light of the fact that most bone matrix macromolecules are glycosylated. The high level of carbohydrates demonstrated in cholesteatoma may significantly affect the proteolytic cleavage of the extracellular bone matrix adjacent to cholesteatoma.

The exact pathogenesis of cholesteatoma as well as its growth is not completely understood. We hypothesize that the level of MMP-9 and TIMP-1 expressions in cholesteatoma as well as MMP-9 and TIMP-1 levels in the sera of cholesteatoma patients might play an important role in better understanding the specific behaviour of cholesteatoma.

Therefore, it seems reasonable to evaluate the expression of MMP-9 and TIMP-1 in cholesteatoma tissues with the correlation between the activity of the selected enzyme and its inhibitor in the sera and plasma of cholesteatoma patients.

Material and methods

Patients

A group of 50 subjects was analyzed. All patients were divided into two groups: cholesteatoma patients (the study group) and the comparison group. The study group (25 patients with cholesteatoma: 10 women and 15 men, aged 22-68 years, mean 48,0 years) underwent surgery between 2010 and 2013. We estimated all cholesteatomas as acquired cholesteatoma according to the classification of Tos as attic cholesteatoma (23), sinus cholesteatoma (2) and tensa cholesteatoma (0) (Tos and Lau, 1989).

The comparison group consisted of 25 patients (4 women and 21 men, aged 20-59 years, mean 34,52) selected for septoplasty due to nasal septum deviation. Patients who suffered from liver diseases, tumor or thrombophlebitis were excluded from the study. Before the surgery, the blood specimens were collected from the study and control groups. All patients signed a written

agreement to take a sample of blood as well as a biopsy of cholesteatoma and retroaricular skin specimens. The study has been approved by the Local Ethic Committee (MMP-9 and TIMP-1 R-J-002/125/2011).

Cholesteatomas samples were taken from the patients during surgery due to chronic otitis media and served as samples of the study group. Macroscopically normal retroauricular skin (3x2 mm) specimens were taken from the same patients during the same surgery (control group).

Biochemical analyses

Blood samples were collected from each patient before surgery using the S-Monovette blood collection system. Ten mL of venous blood samples with anticoagulant (lithheparin) were instantly centrifuged first for 15 minutes x 1000 g and later for 10 minutes x 10000 g at 2-8°C to receive plasma. To obtain serum, the blood samples were left to clot before centrifugation for 15 minutes x 1000g. To standardize clotting conditions, all sera were separated within 1 hour after blood collections. Serum and plasma samples were aliquoted to polypropylene tubes of 500 μL and stored at ≤-80°C until analysis.

Serum and plasma concentrations of MMP-9 and TIMP-1 were measured using the enzyme-linked immunosorbent assay kits (Elisa) (R&D Systems, Abingdon, England), according to the manufacturer's instructions. To analyze the concentrations of MMP-9, the sera samples were diluted 100-fold, and plasma samples 40-fold. Serum and platelet-poor plasma samples were diluted 100-fold before determination for TIMP-1. The manufacturer of assay kits referred to the intra-assay coefficient of variation (CV %) as 1.9% at MMP-9 mean concentration of 2.04 ng/mL, SD=0.039 and as 3.9% at TIMP-1 mean concentration of 1.27 ng/mL, SD=0.05.

MMP-9 and TIMP-1 expressions analyses in cholesteatoma

Before the immunohistochemistry was performed, each specimen, i.e. cholesteatoma and skin samples, was stained with hematoxylin and eosin (H&E) in order to estimate all layers of epithelium and epidermis as well as cholesteatoma perimatrix and a subepithelial layer of skin specimen. Only specimens with all layers of cholesteatoma and skin were accepted for further evaluation. H&E staining also was used to evaluate the intensity of inflammatory reaction within cholesteatoma and to prove no inflammation within skin specimens.

Immediately after tissue samples were obtained, they were fixed in 4% neutral buffered formalin (24 or 48 hr). Formalin-fixed, paraffin-embedded tissue specimens were cut on a microtome into 4 μm sections, which were then deparaffinized in xylene and hydrated in alcohol. To expose the antigen, the slides were heated for 20 min in EDTA buffer (pH=9.0). The activity of endogenous peroxidase was blocked by incubating the sections in 0.5% hydrogen peroxide. Next, the samples were incubated with monoclonal antibodies: MMP-9 (Mouse monoclonal antibody Matrix Metalloproteinase 9, clone 15W2, Leica Novocastra, Poland) - dilution 1:80, for 60 minutes in room temperature; and TIMP-1 (Mouse monoclonal antibody Tissue Inhibitor of Matrix Metalloproteinase 1, clone 6F6a, Leica Novocastra, Poland) - dilution 1:150, for 60 minutes at room temperature. The reaction was performed using the peroxidase detection system (Leica Novocastra, Poland). Color reaction for peroxidase was observed with DAB chromogen (DAB, Leica Novocastra, Poland).

An immunohistochemical assessment was performed by two independent pathologists. Cytoplasmic immunostaining and stromal immunostaining were observed in skin specimens, cholesteatoma epithelium and in inflammatory cells in cholesteatoma perimatrix. A semi-quantitative method was used to evaluate MMP-9 and TIMP-1 expressions which were defined as follows: negative (0) - lack of reaction; weak (1) - positive immunohistochemical reaction in <25% of cells; medium (2) - positive in 25-50% of cells; strong (3) - positive reaction in >50% of cells. The percentage of MMP-9 and TIMP-1 positive cells was calculated in 500 cells in each preparation, at a magnification of x 400.

Statistical analysis

A preliminary statistical analysis revealed that serum levels of MMP-9 and TIMP-1 had a normal distribution but levels of MMP-9 and TIMP-1 in plasma samples did not follow a normal distribution (Kolmogorov-Smirnov test). Consequently, the parametric and nonparametric statistical analyses were used. The Mann-Whitney U test was used to compare the plasma levels in two groups and the Student Test - to compare the data of serum levels in the study and comparison groups. Differences were considered statistically significant with p values below 0.05. The statistical analyses were performed using STATISICA PL 9.0.

Correlations between the expressions of the respective proteins in cholesteatoma tissue and retroauricular skin were calculated using Spearman's correlation coefficient test (STATISTICA 10). The value of $p<0.05$ was also considered statistically significant.

Results

The median and range of MMP-9 and TIMP-1 in plasma and serum concentration for two groups (a study and a comparison groups) are presented in Fig. 1.

Plasma and serum levels of MMP-9 and TIMP-1

The levels of concentrations of MMP-9 and TIMP-1 in plasma and serum were similar in both groups. The serum concentrations of MMP-9 (median) were higher in the study group compared to the comparison group,

inversely to plasma samples that showed slightly lower concentrations of MMP-9 in the study group compared to the comparison group. Serum concentrations of TIMP-1 were found to be lower in the study group than in the comparison group. Plasma concentrations of TIMP-1 were higher in the study group than in the comparison group. All differences were statistically insignificant (Fig. 1).

To show the correlations between plasma and serum levels of MMP-9 and TIMP-1 in the study group, the preliminary Pearson factor was used. According to that evaluation, there was no statistical correlation between MMP-9 and TIMP-1 in serum samples. We noticed a correlation between MMP-9 and TIMP-1 in plasma samples. The concentration of TIMP-1 in plasma samples in the study group depends on the concentration of MMP-9 in plasma samples (Table 1). If the plasma level of MMP-9 is higher, the level of TIMP-1 is also high. We also used the preliminary Pearson factor to observe the correlation between each enzyme and inhibitor depending on the type of blood sample taken.

No correlation was found (Table 1).

Distribution of MMP-9 tissue expression

Immunohistochemical staining was observed in

Table 1. Correlations between concentrations of MMP-9 and TIMP-1 in the sera and plasma of cholesteatoma patients.

variables analyzed	r	p
Serum MMP-9 vs serum TIMP-1	0.135	0.350
Plasma MMP-9 vs plasma TIMP-1	0.636	0.00
Serum MMP-9 vs plasma MMP-9	0.222	0.122
Serum TIMP-1 vs plasma TIMP-1	0.256	0.073

r: Pearson coefficient, p: statistical significance.

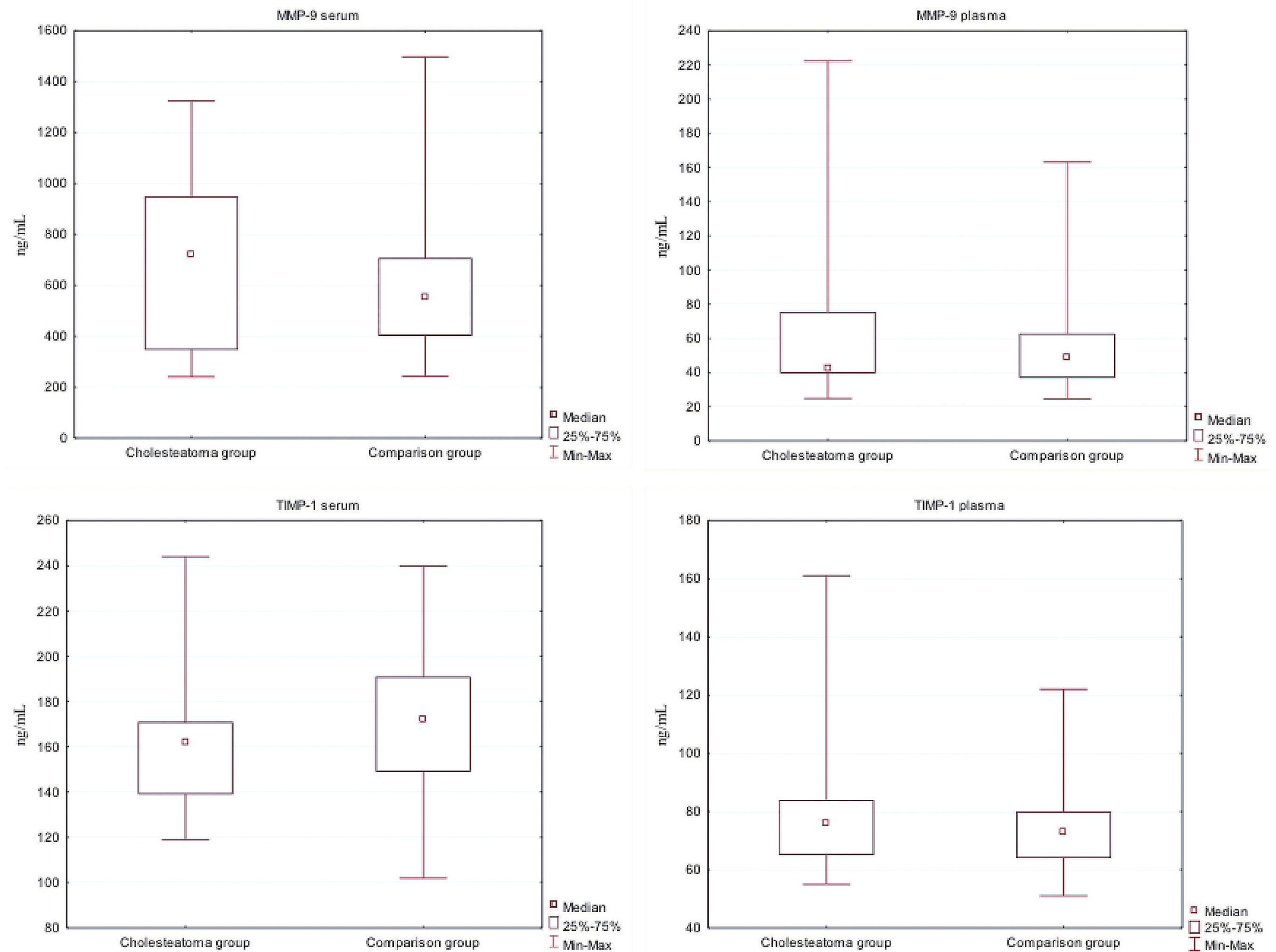

Fig. 1. Median and range of MMP-9, TIMP-1 levels in blood of cholesteatoma patients and a comparison groups.

cytoplasm and stroma in skin specimens, cholesteatoma epithelium and inflammatory cells in cholesteatoma perimatrix.

In cholesteatoma tissue, weak expression of MMP-9 in epithelial cells was observed, while in the stromal layer it was enhanced. The difference in protein expression between the two layers of cholesteatoma was statistically significant (p=0.0006) and it was higher in perimatrix (Fig. 2). The immunohistochemical reaction in skin slices was similar in epithelial and stromal cells, and it was evaluated as weak or medium (Fig. 3). The Comparison of the enzyme expressions in cholesteatoma and in skin slices of retroauricular proved significantly higher expressions of MMP-9 in cholesteatoma perimatrix (p=0.0002). Such a difference was not found in the layer of epithelial tissues tested.

Distribution of TIMP-1 tissue expression

TIMP-1 expression was significantly lower in perimatrix compared to matrix both in cholesteatoma tissue (p=0.01) and in the tissue of the control group (p=0.02) (Figs. 4, 5). TIMP-1 expression was observed

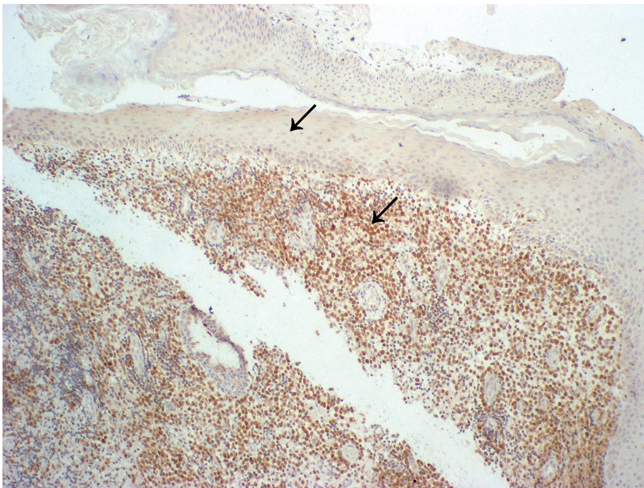

Fig. 2. Matrix metalloproteinase-9 (MMP-9) is shown in perimatrix of cholesteatoma (strong immunoreactivity) and within matrix (weak reactivity). MMP-9 positive cells are marked with arrows. Labeled streptavidin peroxidase method. x 200

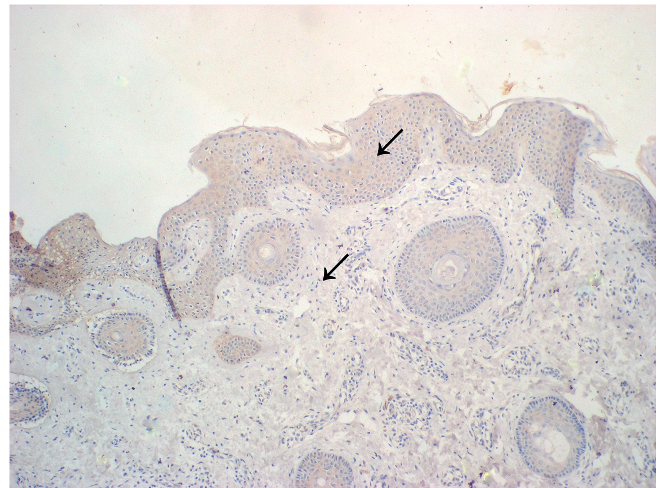

Fig. 3. Matrix and subepithelial connective tissue of retroauricular skin layers show weak positive reaction with MMP-9 (arrows). x 200

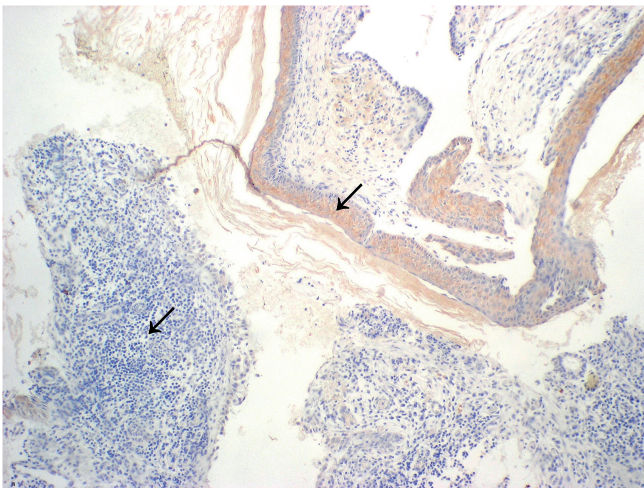

Fig. 4. Tissue Inhibitor of metalloproteinase-1 (TIMP-1) immunostaining in cholesteatoma. Medium positive reaction is observed within matrix (a black arrow). Negative reaction is shown within the area of perimatrix (an arrow). x 200

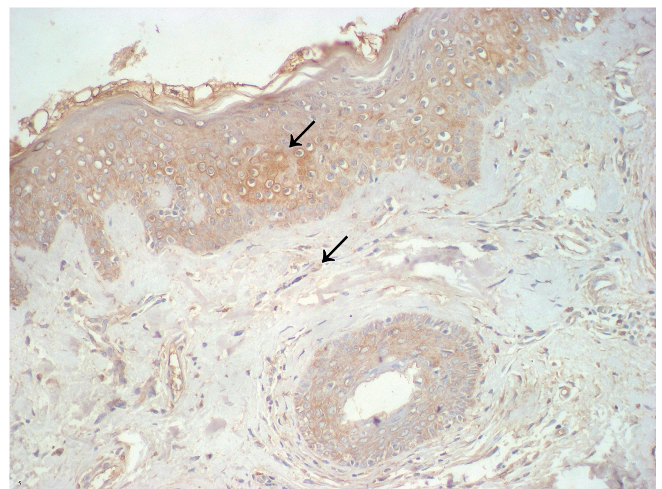

Fig. 5. TIMP-1 immunostaining in retroauricular skin samples. Medium positive reaction within matrix and weak positive reaction in perimatrix (arrows) were observed.

in both epithelium and stroma layers and was higher in the control group (p=0.005). TIMP-1 expression was not observed in cholesteatoma perimatrix in 60% of the examined tissues while in 25% it was rated as poor. This suggests that in cholesteatoma perimatrix, the overexpression of MMP-9 goes together with a decreased expression of this enzyme inhibitory agent, as compared with normal tissue.

Comparison of MMP-9 and TIMP-1 expressions in cholesteatoma tissues

A significantly higher expression of metalloproteinase 9 (p=0.00001) was observed only in the stromal layer, while expression in the epithelium of both MMP-9 and TIMP-1 was comparable. The assessment of the sections of normal skin showed a significantly higher expression of TIMP-1 in epithelial keratinocytes (p=0.03), with no significant difference in the stroma.

Cholesteatoma was always accompanied by inflammation. The inflammatory process correlated with the expression of the proteins tested. The higher the expression of MMP-9, the stronger the inflammation-accompanied cholesteatoma. The color reaction in the assessment of MMP-9 in inflammatory cells was almost always judged as strong. In contrast, TIMP-1 expression was not demonstrated (71%) or was poor (28%) in cholesteatoma tissue. The inflammatory infiltration mainly consisted of mononuclear cells in cholesteatoma perimatrix.

Discussion

In our study, the expression of MMP-9 and TIMP-1 was evident in both the epithelium and cholesteatoma perimatrix. We observed a significantly higher expression in cholesteatoma perimatrix. The difference between MMP-9 and TIMP-1 expressions in cholesteatoma perimatrix was statistically significant, although the difference was not demonstrated in cholesteatoma epithelium, which was not in accordance with Schönermark at al. who showed expression of individual metalloproteinases in all cholesteatoma tissues (Schönermark et al., 1996). The expression of MMP-2 and MMP-9 was observed only within the basement membrane and over the basal epithelium, with no apparent expression in the stroma. The expression of MMP-3 was similar, but some positively stained cells in inflamed tissue accompanying cholesteatoma were isolated. In contrast, the expression of MMP-8 (neutrophil collagenase) was only observed in the stroma as the consequence of the presence of neutrophils. TIMP-1 expression was evaluated as poor in the whole cholesteatoma tissue. There was no expression of MMP-2, 3, 8, 9, and TIMP-1 in the mucosa or in the eardrum of the middle ear. The authors' results are surprising in light of the fact that the studies of other authors showed similar results to ours. In the literature we can find publications showing that MMP-9 and TIMP-1 may have an impact on the development of cholesteatoma and the consequent destruction of the bones (Schmidt et al., 2000; Dornelles et al., 2009; Juhász et al., 2009; Wang and Yu, 2009).

The severity of clinical disease is associated with an aggressiveness of cholesteatoma, and depends, inter alia, on the type of cholesteatoma and its location. Higher expression was demonstrated in congenital cholesteatoma matrix in children as compared with acquired cholesteatoma matrix in adults (Dornelles et al., 2009). This was due to a greater severity of clinical disease and due to associated inflammation and lower patient age. Greater invasiveness is characteristic of the tympanic sinus cholesteatoma as compared with attic cholesteatoma, which was shown in the study of Wang and co-authors. The authors claimed, using immunohistochemical studies, that the expression of MMP tympanic sinus cholesteatoma was higher than in attic cholesteatoma and affected all layers of the epithelium, particularly in the basal membrane (Wang and Yu, 2009). In our study, the tympanic sinus cholesteatoma was present in two cases. Expression of MMP-9 was observed in both the matrix and perimatrix of cholesteatoma. Expression in tympanic sinus stroma cholesteatoma was higher than in cholesteatoma epithelium in the same location. There were no differences between tympanic sinus cholesteatoma and attic cholesteatoma.

MMP-9 is not the only factor involved in the process of resorption of surrounding bone tissue. A correlation between MMP-9 and tenascin (TN) and Ki-67 was proved. These factors are characterized by a higher expression in cholesteatoma with accompanying bone destruction than in the skin of the external auditory canal, and also higher than in cholesteatoma tissue without bone destruction. Increased expression of MMP-9 and Ki-67 was seen in the basal and suprabasal layer of cholesteatoma epithelium and also in the stroma of the connective tissue (Juhász et al., 2009). The results are consistent with our observations, which confirmed the higher expression of MMP-9 in cholesteatoma tissue compared with the skin of the controls. There was no expression of MMP-2, 3, 8, 9, and TIMP-1 in the mucosa or the ear drum of the middle ear. These tissues were from the control group. In our study, the expression of MMP-9 and TIMP-1 was evident in both the epithelium and stroma of cholesteatoma. Unlike Juhász et al., we observed a significantly higher expression in cholesteatoma perimatrix. The difference between MMP-9 and TIMP-1 expression in cholesteatoma perimatrix was statistically significant, while the difference was not demonstrated in cholesteatoma epithelium.

The results of our study are consistent with studies of other authors, who showed that MMP-9 and TIMP-1 may have an impact on the development of cholesteatoma and the consequent destruction of the bones (Schmidt et al., 2000; Juhász et al., 2009; Dornelles et al., 2009; Wang and Yu, 2009).

The reasons for the onset and development of cholesteatoma are not fully understood. Various processes are observed in the course of the disease such as keratinocytes hyperproliferation of epithelium, their apoptosis, neovascularization, stromal infiltration by immunologically competent cells and dysregulation of keratinocytes. Proliferation theory assumes that the ability of endothelial cells of cholesteatoma to proliferate depends on ongoing inflammation that occurs below the epithelium. Molecular markers of epidermal hyperproliferation, such as interleukin 1 (IL-1), transforming growth factor α (TGF-α), epidermal growth factor receptor (EGFR), nuclear antigen of cellular proliferation (PCNA), Ki-67 antigen and cytokeratin are used to explain the specific behavior of keratinocytes in cholesteatoma. The process of keratinocyte proliferation within cholesteatoma epithelium is important not only for understanding the complex mechanisms of the pathogenesis of this disease, but also to anticipate the possible development or recurrence of cholesteatoma (Olszewska et al., 2005; Olszewska and Sudhoff, 2007). Extracellular matrix proteins (ECM) and the basement membrane of the epithelium affect the process of proliferation, migration and differentiation of keratinocytes, among others, by the activity of metalloproteinases (MMPs) in the cell matrix. MMPs are capable of converting of ECM by digestion proteins of this matrix and the basement membrane of the epithelium. They also degrade certain hormones, growth factors, cytokines and chemokines. They take part in the proteolysis of biologically active compounds corresponding to their activation, inactivation or conversion into products with entirely new biological activity (Gomez et al., 1997; Parks, 1999). Recently, the role of epigenetic and miRNA regulation of MMPs has been emphasized (Rutman et al., 2013). There is a SNP (single nucleic polymorphism)- specific regulation of MMP-9 through miRNA targeting the coding region of the gene which allows a better understanding of a multifactorial role of that metalloproteinase (Duellman et al., 2014).

Increased metabolism of proliferating epithelial cells stimulates angiogenesis. This process contributes to the development and spread of cholesteatoma. MMPs can have both stimulatory and inhibitory effect on neovascularization in the process. This happens as a result of the degradation of collagen type I, IV and fibronectin by MMPs, which leads to a relaxation of the structure of the ECM. Epithelial cells, which initiate the formation of new blood vessels, can then penetrate into the stroma. However, MMPs are capable of inhibiting angiogenesis by digestion products of collagen IV, XVII, plasminogen and perlecan. These include angiostatin, endostatin and tumstatin (Iozzo et al., 2009). In contrast, inhibitors of endogenous metalloproteinase restrain neovascularization by suppressing VEGF (vascular epithelial growth factor) (Qi et al., 2003).

It is believed that the process of apoptosis of epithelial keratinocyte of cholesteatoma contributes to cyst enlargement due to the accumulation of residues of the keratin (Olszewska et al., 2006, 2013). MMPs affect both inhibitory and stimulating effect on cell apoptosis. The inhibitory effect of this process is associated with the inactivation of the Fas receptor and the stimulation of the release of growth factors such as epidermal growth factor (EGF) and insulin- derived growth factor (IGF) (Kulik et al., 1997; Gialeli et al., 2009). However, certain MMPs can induce a pro-apoptotic mechanism by actions depending on laminin degradation and impact on signal pathways involving integrins (Egeblad and Werb, 2002).

One of the features of the destructive nature of cholesteatoma is bone resorption in the area adjacent to the cholesteatoma perimatrix. Bone resorption is stimulated, inter alia, by these factors. However, various groups of enzymes, including the metalloproteinases, seem to play a role in the course of the disorder. Therefore, studies on the level of enzyme activities in cholesteatoma and in sera or plasma derived from the blood of patients with this disease contribute to a better understanding of the pathogenic mechanisms of complex chronic otitis media with cholesteatoma. MMP-9, called collagenase B, is produced by most cells of the connective tissue, such as fibroblasts and leukocytes and macrophages. This enzyme, in physiological conditions, is involved in the regulation of blood platelets promoting aggregation independent of thromboxane A2. It plays an important role in regulating the activity of cytokines and chemokines and it also increases the bioavailability of pro-angiogenic growth factors called the "angiogenic switch" (Bergers et al., 2000; Xu et al., 2001; Zaman et al., 2006).

Synthesis of MMPs may be regulated by various factors. EGF, VEGF, TNF-α and IL-1 stimulate the production of MMPs. Blocking agents are the tissue inhibitors of metalloproteinases (TIMPs), α2-macroglobulin, interferon gamma (INF-γ), IL-4 and steroids (Egeblad and Werb, 2002).

TIMP -1 connects with the MMP-9 and haemopexin domain, which is necessary for correctly recognizing the substrate in the enzyme. The primary role of TIMP-1 is the inhibition of proteolytic activity of metalloproteinases. TIMP-1 is involved in determining the balance between the synthesis and degradation of extracellular matrix components in both physiological and pathological situations. TIMP-1 production is induced by growth factors (ß-FGF, PDGF, EGF), cytokines (IL-6, IL-1, Il-1β) and erythropoietin (Nagase and Woessner, 1999; Lambert et al., 2004). Overexpression was evident in both the matrix and the perimatrix of cholesteatoma. Our study on the evaluation of the activity of other markers of proliferation in cholesteatoma also confirmed participation of Ki-67 and PCNA. Proliferative activity was mainly observed in cholesteatoma epithelium (Qi et al., 2003; Iozzo et al., 2009).

In cholesteatoma perimatrix the released cytokines affect epithelial cells causing an increase in MMP-9

release by keratinocytes which was confirmed by the study of Schmidt et al. (2000, 2001). The authors compared the effect of individual elements of cholesteatoma on the release of metalloproteinases by keratinocytes. They showed that keratin, bacterial endotoxin and cholesterol have no direct impact on the synthesis of MMP-9. They suggest that inflammatory cells present in perimatrix induce MMP-9 release through the release of cytokines (IL-1α, IL-1β, TNF-α, TNF-β). This may explain the higher expression of MMP-9 in perimatrix than in cholesteatoma matrix, which we observed in our study.

It has been reported that the expression of MMP-9 and TIMP-1 exists both in the epithelium and stroma of cholesteatoma (Schmidt et al., 2000, 2001; Juhász et al., 2009). More often, however, higher expression is observed in the matrix layers, particularly in the basal layer and suprabasal of cholesteatoma epithelium (Schönermark et al., 1996; Dornelles et al., 2009; Wang and Yu, 2009). In our study, we observed the expression of MMP-9 and TIMP-1 in both the epithelium and stroma of cholesteatoma but a statistically significant difference was only obtained between the study group and control group within perimatrix and it was higher in cholesteatoma. Comparing the cholesteatoma matrix and perimatrix, expression of MMP-9 was significantly higher in perimatrix than in the matrix. This is consistent with Schibosawa and co-workers who demonstrated, using immunohistochemistry stains, the expression of MMP-9 in cholesteatoma tissue compared with the skin of external ear canal and tympanic membrane. The expression of MMP-9 in their studies was observed only in the stromal layer of cholesteatoma and it was closely connected with the occurrence of inflammation and infiltration of cells involved in the inflammatory process. These cells were identified using monoclonal antibodies such as macrophages and neutrophils. There was no expression of MMP-9 in the epithelium of cholesteatoma. Shibosawa et al. suggested that MMP-9 played a role in the development of cholesteatoma by its interfering reactions on the road of cell - extracellular matrix (Shibosawa et al., 2000).

As far as we know there is no current literature concerning MMP-9 and TIMP-1 serum and plasma levels in cholesteatoma patients. MMPs have excessive activity in many physiological and pathological conditions such as cancer, autoimmune and cardio-vascular diseases, as well as Alzheimer's disease (Egeblad and Werb, 2002; Rydlova et al., 2008). Elevated concentrations of the enzymes in a number of cancers correlated with stage, invasiveness, metastases, and 5-year-survival. Higher levels of MMP-9 and TIMP-1 were demonstrated in the sera obtained from the blood of patients with gastric, pancreatic and esophageal cancer, compared with healthy subjects. It was found that the determination of the level of TIMP-1 in plasma patients with gastric cancer may be a better predictor than the level of TIMP-1 in the sera obtained from the blood of these patients. However, in the case of colon and rectal cancer greater clinical application was obtained in the evaluation of TIMP-1 levels than in MMP-9 levels in the diagnosis of this disease (Lukaszewicz-Zajac et al., 2009; Mroczko et al., 2009a,b). In our study, no increase in concentration of MMP-9 and TIMP-1 was achieved in plasma and serum obtained from the blood of patients with cholesteatoma in relation to the control group. One would assume that the inflammation inherent in cholesteatoma is confined to the temporal bone. The results of these tests are, so far, the only reports in the literature.

Conclusion

It seems reasonable to say that cholesteatoma may be limited to the middle ear or parts of the temporal bones. The increased activity of MMP-9 and TIMP-1 in serum and plasma obtained from the blood of patients suffering from cholesteatoma was not observed compared to the activity of investigated proteins in cholesteatoma tissue. Our findings suggest better clinical usefulness of MMP-9 and TIMP-1 expressions in cholesteatoma than either serum or plasma level of these proteins. It might suggest that the higher the expression of MMP-9, the stronger the inflammation- accompanied cholesteatoma.

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