Gene expression profile of collagen types, osteopontin in the tympanic membrane of patients with tympanosclerosis

Monika Sakowicz-Burkiewicz¹, A, C–F, Jerzy Kuczkowski², B, E, Tomasz Przybyła¹, B, C, E, Marzena Grdeń¹, C, E, Anna Starzyńska³, B, F, Tadeusz Pawelczyk¹, A, C, F

¹ Department of Molecular Medicine, Medical University of Gdańsk, Poland
² Department of Otolaryngology, Medical University of Gdańsk, Poland
³ Department of Orofacial and Dental Surgery, Medical University of Gdańsk, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article

Abstract

Background. Tympanosclerosis is a pathological process involving the middle ear. The hallmark of this disease is the formation of calcium deposits. In the submucosal layer, as well as in the right layer of the tympanic membrane, the calcium deposits result in a significant increase in the activity of fibroblasts and deposition of collagen fibers.

Objectives. The aim of our study was to examine the expression level of genes encoding collagen type I, II, III and IV (COL1A1, COL2A1, COL3A1, COL4A1) and osteopontin (SPP1) in the tympanic membrane of patients with tympanosclerosis.

Material and methods. The total RNA was isolated from middle ear tissues with tympanosclerosis, received from 25 patients and from 19 normal tympanic membranes. The gene expression level was determined by real-time RT-PCR. The gene expression levels were correlated with clinical Tos classification of tympanosclerosis.

Results. We observed that in the tympanic membrane of patients with tympanosclerosis, the expression of type I collagen is decreased, while the expression of type II and IV collagen and osteopontin is increased. Moreover, mRNA levels of the investigated genes strongly correlated with the clinical stages of tympanosclerosis.

Conclusions. The strong correlations between the expression of type I, II, IV collagen and osteopontin and the clinical stage of tympanosclerosis indicate the involvement of these proteins in excessive fibrosis and pathological remodeling of the tympanic membrane. In the future, a treatment aiming to modulate these gene expressions and/or regulation of the degradation of their protein products could be used as a new medical approach for patients with tympanosclerosis.

Key words: osteopontin, tympanosclerosis, collagen types

DOI
10.17219/acem/68984

Copyright
© 2017 by Wroclaw Medical University
This is an article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc-nd/4.0/)
Tympanosclerosis is a chronic disease of the tympanic membrane and middle ear manifested by the accumulation of collagen in the elastic and fibrous layer of the lamina propria of the tympanic membrane, submucosal membrane of the tympanic cavity, auditory ossicles and mastoid cavity. The disease process most often refers to the eardrum.1 Typanosclerosis limited to the tympanic membrane is called myringosclerosis and occurs in 24–82% of patients with tympanosclerotic lesions.1,2 The initiation of the immune response leads to the formation of deposits of hyaline with subsequent calcification and sometimes ossification of tympanosclerotic foci.3,4 These changes often lead to restricted mobility of the eardrum and ossicles, resulting in hearing loss. The tympanosclerotic plates formed from deposits of calcium, collagen fibers and hyaline masses result in conductive and mixed hearing impairment, by reducing the mobility of hearing elements, or in rare cases through the perforation of the eardrum, which exacerbates the hearing loss. Typanosclerosis is characterized by dynamic ischemia and stiffening, and even the disappearance of the middle ear tissue.1,5

The etiology of tympanosclerosis is still not fully understood. A widely-accepted consensus recognizes the tympanosclerotic changes as a complication of acute inflammation or injury within the middle ear.2,6–10 A special role is attributed to otitis media with effusion, particularly if patient treatment involved the insertion of a catheter vent.11,12 Other factors that are taken into account in the etiology of tympanosclerosis include immunological processes, genetic predispositions, hypertension and hypercalcemia.13–16

Under the influence of cytokines and other regulatory factors, the fibrosis is initiated in the lamina propria of the tympanic membrane, involving degradation and vacuolization of fibrocytes. This results in the disintegration of cells and accumulation of deposits in the spaces between the collagen fibers. Endoplasmic reticulum released from the destroyed cells is equipped with calcium-binding receptors. This leads to an accumulation of calcium deposits and eventual calcification of tympanosclerotic foci.17 The activated immune cells as well as activated fibroblasts promote tissue remodeling. Our previous in vitro study on fibroblasts isolated from tympanosclerotic lesions demonstrated an up-regulated sensitivity of these cells to mast cell stimulation, which could significantly contribute to the ongoing fibrosis and pathological remodeling of the tympanic membrane.18

Our present study aimed to evaluate the expression level of genes encoding type I, II, III and IV collagen, and osteopontin in the tympanic membrane of patients with tympanosclerosis. Moreover, we determined the correlations between the gene expression levels and clinical stages of tympanosclerosis.

Material and methods

Material

Tymanic membranes were obtained from 25 patients with tympanosclerosis who underwent tympanoplasty in the local Department of Otolaryngology of the Medical University. Patients with accompanied inflammatory disease, with immune deficiency or those taking antibiotics or glucocorticosteroids within the last two weeks were excluded. The clinical stage of the tympanosclerotic lesion was assigned based on the intraoperative evaluation of tympanosclerotic changes according to a modified Tos classification as described previously.19 Morphologically normal tympanic membranes were dissected from 19 people who had died suddenly. The institutional review board at the Medical University previously approved all procedures (NKEBN/432/2009), and written consent was obtained from all patients. Immediately after resection, tissues were placed in RNAlater stabilization solution (Thermo Fisher Scientific, Walther, Massachusetts, USA) and stored at -20°C until the isolation of RNA.

Isolation of total RNA

Isolation of total RNA was carried out in accordance with the Chomczynski procedure, with our own modifications.20 A TRI reagent and suspended material were vortexed briefly and then left standing for 10 min at 4°C. Next, chloroform (250 μL) was added, and the samples were vigorously shaken, incubated at 4°C for 15 min and centrifuged (10,000 × g for 15 min at 4°C). The upper aqueous phase was removed into a new Eppendorf tube, an equal volume of isopropanol was added and RNA was precipitated by overnight incubation at -20°C, followed by centrifugation (10,000 × g for 15 min at 4°C). RNA pellets were washed first with 70% (v/v) ethanol, air-dried and resolved in diethyl-pyrocarbonate-treated thermo-sterilized water, and stored at -20°C until further analysis.

mRNA level determination

The gene expression level was determined by real-time PCR performed in a Light Cycler 480 II (Roche Diagnost GmbH, Manhein, Germany) using Path-IDTM Multiplex One-Step RT-PCR Kit and appropriate Universal ProbeLibrary Set, Human (Roche Applied Science). Transcript levels were normalized to that of the β-actin gene (ACTB). The primer sequences, TaqMan probes and cycling conditions used are listed in Table 1.

Statistical analysis

Statistical analysis was performed using STATISTICA v. 12.0 (StatSoft, Inc., Tulsa, USA). The level of gene expres-
Reverse transcription: 48°C (10 min), 95°C (10 min).

**Results**

Tympanosclerosis (TS) is a chronic disease that is encountered at any age, but usually occurs in the years 30–50. However, as many as 87% of patients are over 40 years old. The pathogenesis of TS is not clear. It usually develops following middle ear infection during the resolution phase of chronic otitis media. The clinical observations indicate that there are some differences in the disease procession between old and young patients. Tympanosclerosis in children is associated with secretory otitis media and the lesions are mostly limited to the tympanic membrane, whereas the tympanosclerotic changes in the elderly are also observed in other middle ear sites: the ossicular chain or the mastoid cavity. These changes are often accompanied by perforation of the tympanic membrane, varying degrees of destruction of the ossicles and accompanied diseases, such as hypercalcemia, hyperlipidemia or hypertension and atherosclerosis. The risk of TS especially increases in children who had ventilation tubes inserted. The clinical observations indicate that the frequency of myringosclerosis is much higher in tympanic membranes with tympanostomy tube insertion than in tympanic membranes without perforation; group II – tympanosclerosis with/ or without perforation. We observed a much lower mRNA level of the COL1A1 gene in patients with stage II of tympanosclerosis. Expression of the COL1A1 gene was inversely correlated with the degree of tympanosclerotic changes (Spearman’s rank correlation (R) = 0.82, p < 0.05, Fig. 2A). Moreover, increased expression of COL2A1, and SPP1 genes strictly correlated with the severity of the disease (R = 0.8 and R = 0.82, p < 0.05, respectively, Fig. 2C, D). Interestingly, the mRNA level of the COL4A1 gene, which was not different in the study group compared to the control group, was correlated with the degree of tympanosclerotic changes (R = 0.48, p < 0.05, Fig. 2B).

**Discussion**

Table 1. Primers and TaqMan probes and cycling conditions used for RT-PCR

<table>
<thead>
<tr>
<th>Gene transcript</th>
<th>Primers</th>
<th>TaqMan probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1A1</td>
<td>(F) agggtacctggacctaag (R) ggaaccaccccttcctca</td>
<td>Universal ProbeLibrary Probe # 67 (Roche)</td>
</tr>
<tr>
<td>COL2A1</td>
<td>(F) tgtgctgacggtcag (R) ccaagcttcaagttcac</td>
<td>Universal ProbeLibrary Probe # 4 (Roche)</td>
</tr>
<tr>
<td>COL3A1</td>
<td>(F) acgtgtaaggtggtcag (R) ccttgagcggagacac</td>
<td>Universal ProbeLibrary Probe # 18 (Roche)</td>
</tr>
<tr>
<td>COL4A1</td>
<td>(F) tgtgacagctgccagcag (R) ggttcaccctttggacctg</td>
<td>Universal ProbeLibrary Probe # 81 (Roche)</td>
</tr>
<tr>
<td>SPP1</td>
<td>(F) cccccagaccttcccaagt (R) gatactacactctggc</td>
<td>Universal ProbeLibrary Probe # 81 (Roche)</td>
</tr>
</tbody>
</table>

Reverse transcription: 48°C (10 min), 95°C (10 min). Amplification: 95°C (10 s), 60°C (45 s).

In a normal tympanic membrane (control) and the tympanosclerotic samples were analyzed with a nonparametric Mann-Whitney U test. The correlations between the level of gene expression and clinical classification of tympanosclerosis were analyzed using Spearman’s R ratio.
Fig. 1. Altered expression of genes (COL1A1, COL2A1, COL3A1, COL4A1) encoding collagens: type I (A), type II (B), type III (C), type IV (D), and gene (SSP-1) encoding osteopontin (E) in sclerotic lesion of tympanic membranes (TTM) and normal tympanic membranes (NTM). The data is means from at least 3 independent measurements performed on isolated total RNA from tympanic membranes, *p < 0.0007; **p < 0.002; ***p < 0.00004.

M. Sakowicz-Burkiewicz, et al. Collagen and osteopontin in tympanosclerosis

Aliens body reaction, inflammation, fibrous hyperplasia, hemorrhage and the release of free hemoglobin between the layers of the tympanic membrane are considered as factors involved in the development of myringosclerosis following tympanostomy tube insertion.25,29,34

Typically, tympanosclerotic changes proceed via the destruction of connective tissue followed by fibrosis resulting in elevated deposition of collagens and subsequent calcification of tissues in the middle ear. A recent histological study demonstrated that a healthy human tympanic membrane consists of collagen type I, II, III and IV.35 All these collagen types have different mechanical properties. The type I collagen fibers are resistant to force, and type II fibers are resistant to deformation. The type III collagen fibers are flexible and elastic, whereas type IV collagen provides support and transport.36

Earlier studies showed that an autoimmune reaction (both cellular and humoral) to type II collagen, an essential component of ear tissue, leads to, among other things,
Osteopontin (OPN), also known as bone sialoprotein I, is a universal regulator of inflammation, biomineralization and tissue remodeling. Osteopontin is expressed by a variety of cell types including fibroblasts, osteoblasts, osteocytes, odontoblasts, hypertrophic chondrocytes, dendritic cells and macrophages. The elevation of OPN level accompanies the exposure of cells to pro-inflammatory cytokines (e.g. TNFα, IL-1β, TGFβ). Several studies indicate that OPN is also up-regulated at sites of pathologic, ectopic calcification. A study by Makiishi-Shimobayashi et al. suggested that macrophage-derived increased expression of SPP1 in inflammatory tissues of the middle ear is involved in the development of tympanosclerosis. Our present study showed that the expression of SPP1 is significantly higher in tympanosclerotic foci and positively correlated with the degree of tympanosclerosis changes. Therefore, the manipulation of local OPN levels may be useful in the treatment of tympanosclerosis.
Conclusions

Tympanosclerosis is a result of post-inflammatory fibrosis characterized by elevated deposition of collagens, and calcification. The present study, with a detailed analysis of the expression of collagen types and osteopontin during the tympanosclerotic process, shows that in the tympanosclerotic membrane the expression of type I collagen is decreased, and the expression of type II and IV collagen and osteopontin is increased.

The altered secretory phenotype of cells from the middle ear induces histological remodeling of the tympanic membrane and correlates with the progression of tympanosclerosis.

References