Influence of Matrix Metalloproteinases content on eardrum atrophy in Otitis Media with Effusion in children

Lylou Casteil[1], Corentin Affortit[2], Jean Luc Puel[1], Fabian Blanc[1], Frederic Venail[1], Jing Wang[1], and Michel Mondain[1]

[1]Université de Montpellier
[2]Institut des Neurosciences de Montpellier

September 21, 2022

Abstract

Objectives Otitis media with effusion (OME) is a common pathology in children. Effusions contain Metalloproteinases (MMPs), which can lead to atrophy of the tympanic membrane (TM) due to destructive effect on the lamina propria. Not all cases of OME are complicated with atrophy, maybe explained by an inter-individual variation of MMP concentration in effusions. The purpose of this study was to determine the correlation between concentration of MMPs and existence of TM atrophy in children. Participants - Main outcome measures The effusion from thirty middle ears were collected during insertion of VT in children aged 15 months to 10 years, including 11 eardrums with tympanic atrophy and 19 without tympanic atrophy. ELISA tests were used to measure concentrations of MMP-2, MMP-7, MMP-9 and TIMP-2 in the effusions. Correlations between MMP levels and atrophy of eardrum was investigated, as well as correlation with age, gender, number of previous ventilation tube insertion, and viscosity of the glue. Results The mean concentration of MMP-2 was higher in the atrophic group than in effusions without TM atrophy (0.6 ng/mg versus 0.5 ng/mg total protein respectively), while the TIMP-2 concentration was lower in this group. The level of MMP-2 decreased with the age. Finally, a significantly higher concentration of MMP-9 and TIMP-2 was found in high-viscosity effusions. Conclusion This study suggests that MMP-2 activity could play an important role in destruction of the eardrum during OME in infancy. MMP-2 level assessment could be interesting for determining children with risk of lamina propria destruction.

INTRODUCTION

Otitis media with effusion (OME) is a chronic otitis media with inflammatory effusion in the middle ear. Inflammation causes a thickening of the mucosa within the middle ear cleft which, by modifying the gas exchange capacities, is responsible for a decrease in the partial pressure of oxygen facilitating the retraction of the tympanic membrane (TM) (1). In children, inflammation often arises via an infectious origin by contamination of middle ear cavities via the Eustachian tube.

The diagnosis of OME is clinical, by otoscopy and by tympanometry (2). The exact incidence of OME is unknown, due to the nature of the disease which can be asymptomatic. However, it is thought to be up to 50% children under 12 months of age and 60% children at the age of 2 years. A study conducted with systematic screening of children aged 1 to 4 years suggests a prevalence between 15 and 40% (3).

OME can lead to structural changes in the TM (4)(17). Tympanosclerosis plaques are classic and not dangerous, but atrophy of the eardrum can lead to retraction pockets, and then into more significant disease such as cholesteatoma or chronic adhesive otitis media (5) (6). In otoscopy, atrophy appears as a thin, partially or totally transparent eardrum (8). Histologically, the atrophy of the pars tensa is explained by the destruction of type IV collagen fibers of the lamina propria (7), and it has been suggested that the activity of matrix metalloproteinase are involved in destruction of tympanic membrane fibrous layer (9).

An increase in matrix metalloproteinase (MMP) activity may be present in OME. MMPs are a multigenic family (25 members and 7 different classes) of zinc-dependent, secretory or membrane enzymes. They control the activity of extracellular matrix proteins by proteolytic cleavage. Their targets include membrane-bound or soluble molecules involved in the transmission of intercellular signals such as cytokines, chemokines, trophic factors, adherence proteins and different receptors. Correlation has been demonstrated between significant activity of MMP-2 and 9 (gelatinases), MMP-3 (stromelysin 1) and 7 (matrilysin 1) and MMP-8 (collagenase-2) and the viscosity of the effusion (7) (10) (11) (12). Each MMP is specific to one or more component of the extracellular matrix (13). Gelatinases (MMP-2, MMP-9) are the fourth class of MMPs, and their proteolytic activity is directed against denatured interstitial collagen (gelatin) and type IV and V collagens of basal membranes. MMP-9 (gelatinase-B) expression is low or absent in normal tissues and limited to monocytes and macrophages. MMP-7 (matrilysin-1) is part of the matrilysin group, and is specifically expressed by tumoral epithelial cells. Its proteolytic spectra are partially divergent but include fibronectin and gelatin.

In tissues, the proteolytic activity of MMPs is controlled by four inhibitors called TIMPs (tissue inhibitors of metalloproteinases) (14). TIMPs (TIMP-1 to TIMP-4) are endogenous inhibitors of activated MMPs. The MMP/TIMP system controls the cell-cell and cell-matrix interactions involved in many physiological processes, including proliferation, differentiation, migration and cell death. TIMP-2 works as an MMP inhibitor but also as an activator since it inhibits the active form of MMPs and activates proMMPs. Thanks to their C-terminal domain, there is a specificity between MMP and TIMP types, TIMP-2 is an inhibitor that binds specifically to MMP-2.

In France, insertion of VT is the second most frequent ENT surgery procedure after tonsillectomies and/or adenoidectomies. This surgery is indicated for auditory and/or infectious reasons and/or OME complicated with TM atrophy with posterior mesotympanic retraction (2). Studies are inconclusive about the effectiveness of VTs on the development of tympanic atrophy (15) (16). The inter-individual variability of the lytic activity of OME liquids, both in its composition and/or enzymatic concentration, could explain the difficulty to statistically demonstrate the efficacy of VT in preventing the development of atrophy.

The purpose of this study was to assess a correlation between the presence of eardrum atrophy and the level of MMP-2, MMP-9, MMP-7, TIMP-2 in OME effusion. The secondary objective was to determine if there is a relationship between the level of MMPs and TIMP-2 and the patient's age, gender, history of VT insertion, thickness of the glue (mucous or serous), and hearing loss.

METHODS

This is a pilot study and it is prospective, monocentric, observational and case-controlled.

Population

Thirty-five children, 12 girls and 23 boys, whose indications for VT were selected according to SFORL recommendations (French ENT Society)(18), were included in this study (Figure 1).

Five children were excluded (4 for insufficient volume to allow analysis, 1 for blood contamination of the effusion). Of the 30 remaining children, a collection of 30 OME fluid samples was collected, 11 from ears with tympanic atrophy (preoperative examination under microscope done by two different examiners) and 19 from ears without tympanic atrophy (preoperative examination performed by two different examiners finding thinness from visual aspect under microscope).

For each patient, the effusion was collected from both ears prior to VT insertion but only one ear was analysed per child. In case of unilateral TM atrophy, only this sample was analysed. In the event of bilateral atrophy or healthy bilateral eardrum, the laterality of the effusion to be analysed was selected by a coin toss.

The study population ranged from 15 months to 10 years of age. Eighteen right ears and 12 left ears were selected. There were no differences (Chi-2 test with Prism's Biostatgv and Graphpad software) between the two groups (healthy and atrophic eardrums) in gender ($p=0.28$), age ($p=0.881$), audiometric loss ($p=0.299$), or number of VT insertions in the history ($p=0.62$ with the chi2 test and with a Cramer $V=0.01$). The

effusion was more mucous than serous in both groups (serous in 18% of cases in atrophic patients and 15% in healthy patients p=0.865).

Liquid collection protocol

The procedure was performed under general anaesthesia. After cleaning and washing the ear canal with saline solution, each effusion was collected by aspiration with a 1.2 gauge suction cannula connected to a cell trap, following myringotomy. The glue was considered mucosal if it was thick, difficult to aspirate and stuck to the wall of the cell trap (19) (requiring micro forceps to collect the effusion if necessary). It was considered serous if the suction was easy and the collection was liquid and mobile in the cell trap.

The samples collected were immediately immersed in a container filled with ice to prevent protein denaturation by heat, and then transported immediately to the laboratory. Each mucoid effusion was separated into two samples, using micro scissors, and then placed in two cryotubes marked with the patient's anonymised code. The thinner serous samples were divided into two cryotubes by pipette. No dilution was carried out at this stage. The samples had an average volume of 200µl. The cryotubes were frozen at -80°C.

Dosing protocol - ELISA technique

An ELISA kit was used (R&D system Quantikine ELISA) for a quantitative analysis of the total MMP-2, MMP-7 and MMP-9 (free and complex forms) and human TIMP-2 (plate of 96 wells pre-coated with the primary antibody). Conditioning was performed using a saline / EDTA buffer in order to obtain viscosity of the samples to be compatible with this technique. Samples were diluted in a buffer with 0.1 M NaCl, 1mM PMSF, 1x LAP, 5mM EDTA, 20mM Tris 0.6M. Thorough homogenization of the effusions was achieved using a pestle, to manually extract the proteins. The samples were then placed on a circular stirrer for 1 hour at 4°C. A total protein assay was performed using BCA kits (BCA protein assay kit, ThermoFisher Scientific®) on each buffered sample.

We used 1/10 and 1/20 dilutions of the middle ear effusions for the analysis of MMP-2, TIMP-2 and MMP-7 concentrations, and 1/40 for the determination of MMP-9. On each plate, 64 ELISA wells were used to test the duplicate samples. The remaining 16 wells were used to carry out the standard curve with standard solutions.

Analysis was performed by spectrophotometry at 450 nm and 540 nm. The optical density (OD) was the average of the two ODs obtained by each duplicated sample on the microplate reader. A concentration in ng/mL was obtained (ratio of OD to standard curve)), then this level was normalized by the total protein level of the sample, the final concentration being expressed in ng/µg protein.

Results are presented as an average concentration and standard deviation. The statistical data were calculated using a non parametric test (Mann-Whitney test for independent data).

Ethical and regulatory aspects

This project has been validated by the Local Ethics Committee (IRB of Montpellier) in MR-004. An explanatory note was given to the parents and children included in the study, and consent was collected at the end of the consultation.

RESULTS

Determination of MMP-2, MMP-7, MMP-9 and TIMPS 2 levels (Table 1&2)

The average MMP-2 level was lower in the effusions from non-atrophic TMs (average 85,4 ng/ml) than in the effusions from atrophic TMs (113,2 ng/ml), whether the concentration is expressed in ml of effusion or related to the total protein content: this difference was not significative between the two groups (p=0.28).

The mean concentration of TIMP-2 was higher in healthy subjects (mean 73,7 ng/ml – 0,64 ng/µg total protein) than in the atrophic TMs group (65,8 ng/ml – 0,51 ng/µg total protein). There is no significant difference between the two groups of samples (p=0.76).

The average MMP-7 level was 71 ng/ml in the non-atrophic samples (0,59 ng/µg total protein) vs. 68,5 ng/ml in the atrophic group (0,48 ng/µg total protein): the difference is not significant between the two groups (p=0.68).

The quantities of MMP-9 measured in the samples were higher than for MMP-2 and MMP-7. The average MMP-9 level was 1120 ng/ml in the non-atrophic group (110 ng/µg total protein) vs. 822,6 ng/ml in the atrophic TMs samples (0,48 ng/µg total protein). However, this difference was not significant (p=0.73).

Clinical predictive factors for MMPs and TIMP-2 concentration

MMP-2 and TIMP-2 level are negatively correlated to the age of the child (Figure 2). However, this difference was not significative (p= 0,08) and the ratio MMP-2 / TIMP-2 remains constant among ages. MMP and TIMP-2 rates were not influenced by the gender, or the preoperative conductive hearing loss.

The MMP-7 rate was significantly higher in children who no history of previous VT insertion (p = 0.013). This trend is not significant for MMP-2 and TIMP-2 (p=0.19).

The MMP and TIMP-2 levels were higher in the thicker, more mucous, effusions regardless of the condition of the eardrum. This difference is significant for MMP-9 and TIMP-2 (p=0.0017 and p=0.013 respectively).

DISCUSSION

OME is a major public health problem in paediatric ENT practice (8) and understanding the mechanism of tympanic complications is fundamental in reducing the sequelae of this disease (5). Proteolytic enzymes can play an important role in the development of TM atrophy associated with OME, and then into cholesteatoma. Many authors refer to the role of proteases in the pathogenesis of OME complications (9–11,15,20). Our study suggests a non-significant link between the level of MMP-2 in OME fluid and the existence of tympanic atrophy. A higher concentration of MMP-2 in the effusions of patients with atrophic tympanic membranes has been found. Its specific inhibitor TIMP-2, assessed in order to estimate the active MMP-2 level without the inactive pro-enzymes (14), was also lower in the effusions of patients with atrophic tympanic membranes. The MMP-9 level tended to be lower in children with atrophy, while the MMP-7 rate was quite similar in both groups. So, the MMP-2 enzyme activity tended to be higher in cases of tympanic atrophy, even if the difference was not significant.

The ELISA technique was used for the assays. Gelatin or casein zymography is an electrophoresis examination frequently performed in other studies to measure MMP activity, because it dissociates MMPs from TIMPs. However, ELISA, which is also commonly used for this type of assay, is a validated technique. The MMP concentrations measured in this study in ng/µl were consistent with MMP levels found in previous publications, except for the MMP-9 concentrations which were higher than in other studies. A South Korean study (21) studying MMP-9 levels in OME with and without allergy found also high concentrations ranging from 100 to 920 pg/mg total protein.

Destruction of fibrous layer in OME may be related to the duration of the OME's evolution (5), but this cannot be demonstrated in the general population because OME is often asymptomatic. In the literature, the duration of OME is often estimated from the number of VT insertions, and the age of the patients. In our study, MMP2 and TIMP-2 concentrations tended to decrease with age (figure 2), as reported in a 1994 American study (16).However, the ration MMP-2/TIMP-2 did not vary among age.

In our study, the number of previous VT was not correlated with the MMP level, but the MMP-7 rate which was significantly higher in children who had no prior history of VT placement. The study by Jennings and al. (20) found an increase in MMP-2 levels with the number of VT operations, while a Swedish study by Juhn and al. (16) found no relationship between the number of VT insertions and MMP-2 and 9 concentrations.

In our study, a significantly higher concentration of MMP was found in the thicker mucous effusions, the difference being significative for MMP-9 and TIMP-2 (p=0.0017 – p=0,013 respectively). This result has been demonstrated in other studies (9,10,15,20). Interleukins such as TNFα and IL-1 are present in higher concentrations in mucous effusions (16). The role of these molecules in inducing the transcription of MMPs is consistent with the results of our research. On the other hand, our results for TIMP-2 concentrations are at odds with the results from Moon's work, since we found a significant link between elevated TIMP-2 concentrations and mucous effusions (9).

One of the limitations of our study is assessment of the degree of tympanic atrophy by examination under a microscope, even with an examination under microscope performed by two different examiners. The gold standard would be to perform biopsies of the eardrum (4). However, this could lead to perforations and is questionable in children. In further studies it could be possible to analyse an animal model to quantify the relationship between the degree of eardrum atrophy and MMP concentrations in OME effusion.

The second limitation was the number of children enrolled: inclusion of more subjects would increase the strength of our study. In addition, it would be interesting to compare intra-individual MMP-2 levels in children with unilateral tympanic atrophy, the child being his own control.

CONCLUSIONS

This study showed a higher MMP-2 concentration in children presenting with tympanic atrophy, while the TIMP-2 concentration was lower in this group, suggesting a role of MMP-2 in the digestion of type IV collagen from the basement membrane of the tympanum. This study also suggests a higher concentration of MMP-9 and TIMP-2 in case of thicker, mucous effusions. The level of MMP-2 decreased with the age, suggesting more aggressive OME in young children leading to atrophy. In the future, MMP-2 concentration assessment could refine the indications for the insertion of VT in children to prevent the emergence of cholesteatoma.

REFERENCES

1. Mondain M, Bordure P. Recommandations de Pratiques Cliniques (RPC) de la Société Française d'ORL et de Chirurgie Cervico-Faciale Prise en charge thérapeutique des OSM de l'enfant. 2016.

2. Triglia J-M, Roman S, Nicollas R. Otites séromuqueuses. J Pediatr Pueric. 2004;17(2):83–100.

3. Paradise JL. Otitis Media in Infants and Children. Pediatrics. 1980 May 1;65(5):917–43.

4. Knutsson J, Bagger-Sjöbäck D, von Unge M. Structural tympanic membrane changes in secretory otitis media and cholesteatoma. United States, Lippincott Williams & Wilkins; 2011.

5. Maw AR, Bawden R. Tympanic membrane atrophy, scarring, atelectasis and attic retraction in persistent, untreated otitis media with effusion and following ventilation tube insertion. Int J Pediatr Otorhinolaryngol. 1994;30(3):189–204.

6. Dornelles C de C, da Costa SS, Meurer L, Rosito LPS, da Silva AR, Alves SL. Comparison of acquired cholesteatoma between pediatric and adult patients. Eur Arch Oto-Rhino-Laryngol Off J Eur Fed Oto-Rhino-Laryngol Soc EUFOS Affil Ger Soc Oto-Rhino-Laryngol - Head Neck Surg. 2009 Oct;266(10):1553–61.

7. Knutsson J, Bagger-Sjöbäck D, von Unge M. Collagen type distribution in the healthy human tympanic membrane. United States, Lippincott Williams & Wilkins; 2009.

8. Ryding M, White P, Kalm O. Eustachian tube function and tympanic membrane findings after chronic secretory otitis media. Int J Pediatr Otorhinolaryngol. 2004;68(2):197–204.

9. Moon SK, Linthicum FH, Yang HD, Lee SJ, Park K. Activities of matrix metalloproteinases and tissue inhibitor of metalloproteinase-2 in idiopathic hemotympanum and otitis media with effusion. Acta Otolaryngol (Stockh). 2008 Feb;128(2):144–50.

10. Antonelli PJ, Schultz GS, Kim KM, Cantwell JS, Sundin DJ, Pemberton PA, et al. Alpha 1-antitrypsin and ilomastat inhibit inflammatory proteases present in human middle ear effusions. The Laryngoscope. 2003 Aug;113(8):1347–51.

11. Lauhio A, Rezes S, Tervahartiala T, Sziklai I, Pitkäranta A, Sorsa T. Matrix metalloproteinase-8/collagenase-2 in childhood otitis media with effusion. Ann Med. 2012 Feb;44(1):93–9.

12. Hassan F, Ren D, Zhang W, Gu X-X. Role of c-Jun N-terminal protein kinase 1/2 (JNK1/2) in macrophage-mediated MMP-9 production in response to Moraxella catarrhalis lipooligosaccharide (LOS). PloS One. 2012;7(5):e37912.

13. Fingleton B. Matrix metalloproteinases as regulators of inflammatory processes. BBA - Mol Cell Res. 2017;1864(11):2036–42.

14. Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function 1 1 Dedicated to Professor H. Neurath on the occasion of his 90th birthday. Biochim Biophys Acta BBAProtein Struct Mol Enzymol. 2000;1477(1):267–83.

15. Granström G, Holmquist J, Jarlstedt J, Renvall U. Collagenase activity in middle ear effusions. Acta Otolaryngol (Stockh). 1985 Dec;100(5-6):405–13.

16. Juhn SK, Garvis WJ, Lees CJ, Le CT, Kim CS. Determining otitis media severity from middle ear fluid analysis. Ann Otol Rhinol Laryngol Suppl. 1994 May;163:43–5.

17. Sano S, Kamide Y, Schachern PA, Paparella MM. Micropathologic changes of pars tensa in children with otitis media with effusion. Arch Otolaryngol Head Neck Surg. 1994 Aug;120(8):815–9.

18. Sakakura K, Hamaguchi Y, Harada T, Yamagiwa M, Sakakura Y. Endotoxin and lysosomal protease activity in acute and chronic otitis media with effusion. Ann Otol Rhinol Laryngol. 1990 May;99(5 Pt 1):379–85.

19. Jennings CR, Guo L, Collins HM, Birchall JP. Matrix metalloproteinases 2 and 9 in otitis media with effusion. Clin Otolaryngol Allied Sci. 2001 Dec;26(6):491–4.

20. Jang C-H, Shin S-H, Cho H-H, Moon S-J, Cho Y-B. Expression of matrix metalloproteinase-9 and -2 in pediatric chronic otitis media with effusion. Int J Pediatr Otorhinolaryngol. 2006 Jul;70(7):1155–8.

21. Zielhuis GA, Haggard MP, Broek P van den. Long-term effects of otitis media with effusion: otomicroscopic findings. Am J Otol. 1995;16:365–72.

Hosted file

`MMP MM v5 fig.docx` available at https://authorea.com/users/509751/articles/587052-influence-of-matrix-metalloproteinases-content-on-eardrum-atrophy-in-otitis-media-with-effusion-in-children