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Activities of matrix metalloproteinases and tissue inhibitor of metalloproteinase-2 in idiopathic hemotympanum and otitis media with effusion

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Abstract

Conclusion—The expression profile of matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteinase-2 (TIMP-2) was specific to the type of middle ear effusion. Further studies are necessary for elucidating its correlation with the sequelae of otitis media with effusion (OME) and idiopathic hemotympanum.

Objectives—We aimed to investigate the relative activities of gelatinases (MMP-2 and 9), stromelysin-1 (MMP-3), matrilysin-1 (MMP-7) as well as measuring TIMP-2 levels in the serous and mucous effusions of OME and hemorrhagic effusion of the idiopathic hemotympanum.

Method—Middle ear effusions were collected from patients with OME and idiopathic hemotympanum, and were classified as mucoid, serous or hemorrhagic. MMP activity in the effusion samples was examined by gelatin and casein zymography. Levels of TIMP-2 were measured by ELISA. Human temporal bones sections, with and without otitis media (OM), were examined histologically.

Results—One case showed tympanic membrane thinning in the OM group, but none in the control group. While MMP-2 was present in all effusions, the active form of MMP-2 was found only in mucous effusions. MMP-3 and MMP-7 activity was detected only in the mucous effusions. MMP-9 exhibited activity in all effusions, with the highest levels in mucous effusions. TIMP-2 levels were markedly elevated in serous effusions.

Keywords

Matrix metalloproteinase; Tissue inhibitor of metalloproteinase; Otitis media with effusion; Idiopathic hemotympanum

INTRODUCTION

Otitis media with effusion (OME) is characterized by the chronic accumulation of fluid in the middle ear space with an intact tympanic membrane. OME may occur due to poor Eustachian

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tube function or as an inflammatory response following acute otitis media. It has been reported that OME is associated with tympanosclerosis, atrophy, retraction and atelectasis of tympanic membrane as sequelae [1–3] although there are controversies regarding whether simple OME can lead to structural changes in the tympanic membrane without recurrent inflammation. These changes in the tympanic membrane impact hearing function as well as playing a role in the pathogenesis of cholesteatoma.

Idiopathic hemotympanum (or blue ear drum) is caused by a recurrent hemorrhage in the middle ear or mastoid in the presence of Eustachian tube obstruction [4,5]. Cholesterol granuloma develops when the extravasated blood is broken down to cholesterol or hemosiderin, which subsequently induces a foreign body reaction [6]. Idiopathic hemotympanum can be associated with retraction, bulging, thinning or adhesion of tympanic membrane when occurring with recurrent inflammation [7]. It is possible that protease activity is involved in idiopathic hemotympanum, but it has not been investigated in detail.

There are several mechanisms for the degradation of structural macromolecules of connective tissues and basement membranes. These include the metalloproteinases (MMPs), tissue plasmin, neutrophil serine proteinase, and phagocytic or osteoclastic mediated pathways [8]. Increased activity of MMPs has been demonstrated in middle ear effusions [9,10] and in cholesteatoma tissue [11,12]. It is believed that the altered elasticity of the tympanic membrane could be attributable to the OME sequelae. MMPs are a family of highly homologous metal-dependent endopeptidases that can cleave most of the constituents of the extracellular matrix such as collagen, fibronectin, laminin and elastin [8], and are inhibited by endogenous tissue inhibitor of metalloproteinases (TIMPs) [13] or synthetic inhibitors such as EDTA and phenanthroline. The ratio of activated MMPs and TIMPs is a key determining factor between degradation and biosynthesis of the matrix.

The aim of this study was to investigate the relative activities of MMP-2 and 9 gelatinases, stromelysin-1 (MMP-3), matrilysin-1 (MMP-7) as well as measuring TIMP-2 levels in the serous and mucous effusions of OME and hemorrhagic effusion of the idiopathic hemotympanum.

MATERIAL AND METHODS

1. Collection of middle ear effusions

A total of fifteen middle ear effusions were randomly selected from effusion collections of patients with OME or idiopathic hemotympanum. All aspects of human middle ear effusion collection were performed according to an approved IRB protocol at Ajou University School of Medicine. Effusions were collected after tympanotomy using Juhn Tymp-Tap (Metronic/Xomed, Jacksonville, FL) during inserting the ventilation tube, and divided into three groups (mucous, serous and hemorrhagic) according to color and viscosity [14]. Particularly, when the patients clinically showed the black-blue drum more than six months with a significant conductive hearing loss, the effusions were grouped as hemorrhagic [4]. Collected effusions were stored at −70°C to preserve enzyme activity, and five samples were randomly selected from each group. The age distribution of fifteen patients (4 females and 11 males) ranged from 2 to 58 years with a mean of 17.8 years, and the average duration of OM was 2.1 years. The range of pure tone average was from 27 to 40 dBHL with a mean of 34.3 dBHL. Six of the patients (2 females and 4 males) had a history of ventilation tube insertion more than once (Table 1).

2. Zymography

Total protein concentration of effusions was measured with the Bradford assay. Enzyme activities of MMP-2, 9, 3 and 7 were assessed by zymography using either gelatin (for MMP-2 and 9) or casein (for MMP-3 and 7) impregnated gels. Briefly, 50 μg of total protein was loaded onto 10% SDS-PAGE gels containing 0.1 g/ml gelatin (Sigma, St. Louis, MO) or 1 mg/ml casein (Sigma), and electrophoretically separated at 10 mA for 3 hours under non-reducing conditions. Zymography demonstrates total activities of MMPs since SDS dissociates MMPs from TIMPs [15]. After re-naturing in a mixture of 50 mM Tris pH 7.5, 0.1 M NaCl and 2.5% Triton X-100, the gels were incubated for 17 hours in 50 mM Tris, 10 mM $CaCl_2$ and 0.02% NaN_3 at 37°C. Control gels were incubated in the presence of 10 mM EDTA instead of calcium to rule out serine protease activity. Gels were stained with Coomassie blue and destained in a mixture of 5% acetic acid and 10% methanol. The subtypes of MMPs were identified by the use of molecular weight standards and by running samples of purified MMP-2 and MMP-9 enzymes (Calbiochem, San Diego, CA). Areas of enzymatic degradation were quantified by densitometry and expressed as arbitrary units/50 μg total protein.

3. ELISA

The concentration of TIMP-2 was measured by ELISA. Collected effusions were diluted in the assay buffer containing 0.03 M phosphate, 0.1 M NaCl, 0.3% BSA and 0.01 M EDTA, and TIMP-2 levels were assessed using TIMP-2 Human Biotrak™ ELISA System (Amersham Biosciences, Piscataway, NJ), using a two-site sandwich format. Briefly, standards and samples were incubated in 96 microtiter wells, pre-coated with anti-TIMP-2 antibody. After washing, bound TIMP-2 was detected using a peroxidase-labeled anti-TIMP-2 antibody. Excess reagents were removed by repeated washings and aspirations. The amount of peroxidase bound to each well was determined by the addition of 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) substrate. The reaction was stopped by the addition of the stop solution (sulfuric acid), and the resultant color was read at 450 nm in a microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA). The concentration of TIMP-2 in each sample well was determined by interpolation from a standard curve. Results of TIMP-2 level were expressed as ng/mg total protein, and represented all forms of TIMP-2 since anti-TIMP-2 antibody recognizes both TIMP-2 and MMP-TIMP-2 complex.

4. Archival human temporal bone sections

A total of nineteen temporal bones with OM history and 157 normal temporal bones (negative controls) were selected from the archival human temporal bone bank at the House Ear Institute (Table 2). Temporal bones with a confirmed history of otorrhea were excluded. Temporal bones were fixed in 4% formalin, decalcified in 125 mM EDTA, embedded in celloidin, and sectioned in the horizontal plane at a thickness of 20 μm. Every tenth section was stained with H & E, mounted on a glass slide, and examined by light microscopy. The thickness and morphology were compared in the posterior part of the tympanic membrane. Only one case, among the nineteen cases with OM history, showed a characteristic difference in morphology in the tympanic membrane. Thickness of the tympanic membrane was measured at three comparable sites. This corresponded to a 55 year-old female, having a past history of bilateral recurrent OM as a child. The negative control was a 45 year-old male, who suffered from bilateral progressive sensorineural hearing impairment (Table 3).

5. Statistics

All experiments pertaining to Zymography and ELISA were carried out in duplicate. Results are expressed as mean ± standard deviation. Statistical analysis was performed using Student's *t*-test, with significance considered to be $p<0.01$ or $p<0.05$.

RESULTS

1. Human temporal bone study

To determine if a history of recurrent or chronic otitis media is associated with tympanic membrane thinning, archival human temporal bones were examined by one experienced pathologist. One case showed tympanic membrane thinning out of ninteen OM group, but none in the control group. In this particular case, the tympanic membrane was retracted without perforation, and effusion was found. When measured at three comparable sites, the tympanic membrane was significantly thinner in the case with chronic OM (266.1 ± 13.4 μm) compared to the normal subject (690 ± 39.6 μm) (Table 3). Blood vessels and connective tissue lateral to the manubrium were not seen in cases with OM in contrast to the normal subject (compare Figure 3C with 3A). The fibrous layer of the tympanic membrane showed atrophy and irregularity in cases with OM, which was well preserved in the normal subject (compare Figure 1D with 1B). It is likely that the atrophy or destruction of the fibrous layer by recurrent OM resulted in thinning of the tympanic membrane. In contrast, the tympanic membrane showed a typical conical shape, and connective tissue and blood vessels were seen lateral to the manubrium in the normal subject (Figure 1A). In addition, the fibrous layer between the two epithelial layers was preserved and showed a regular arrangement (Figure 1B).

2. Activity of MMP-2, 3, 7 and 9 in mucous, serous and hemorrhagic effusions

We hypothesized that the activity of MMPs are involved in destruction of tympanic membrane fibrous layer. Previously, increased activity of gelatinases (MMP-2 and 9) has been reported in the mucous middle ear effusion [10], however, we additionally sought to investigate stromelysin-1 (MMP-3) and matrilysin-1 (MMP-7) in three types of middle ear effusions: serous, mucous and hemorrhagic. MMP activity of middle ear effusions was examined using gelatin or casein zymography using substrate-containing gels under non-reducing conditions. After staining of the gels, levels of enzyme activity were measured by quantifying enzymatically degraded areas using densitometry. Gelatin zymography (Figure 2A) demonstrated roughly similar levels of pro-MMP-2 activity (72 kD) in all samples, with markedly higher levels of pro-MMP-9 (92 kD) in the mucous effusions compared to the serous and hemorrhagic effusions. The active form of MMP-2 (66 kD) was found only in the mucous effusions, while the active form of MMP-9 (84 kD) was found in both mucous and hemorrhagic effusions. In contrast, casein zymography (Figure 2B) demonstrated enzyme activities of MMP-3 (29 kD) and MMP-7 (57 kD) only in the mucous effusions. The presence of multiple bands in the zymograms indicates that they must all be due to MMP activity since the presence of EDTA in control gels showed a complete absence of all enzyme activity (Figure 2, lane E). It has been postulated that MMP dimerization and other molecular complex formations may play important roles in the formation of multiple bands [8], however the exact molecular species involved in this process have not been determined. Densitometry of the zymograms (Figure 3A) demonstrated different patterns of enzyme activities depending on the type of effusion. While the levels of MMP-2 activity were not significantly different between the three effusion groups, MMP-9 activity levels were highest in mucous effusions and lowest in serous effusions. Furthermore, activities of MMP-3 and 7 were significantly higher in mucous compared to serous and hemorrhagic effusions.

3. TIMP-2 levels

Since MMP activity is balanced by TIMPs, we explored to investigate the level of TIMP-2 according to the type of middle ear effusions. TIMP-2 levels, expressed as ng/mg total protein, were measured by commercially available two-site sandwich format ELISA kits. The level of TIMP-2 was 331.4 ± 209.4 ng/mg total protein in the serous effusion and 7.1 ± 15.8 ng/mg total protein in the hemorrhagic effusion, but not detected in the mucous effusion (Figure 3B). Since TIMP-2 can sequester MMPs, the high levels of TIMP-2 measured in serous effusions

are likely to diminish the overall activity of MMP-2 *in vivo*. Thus the actual level of MMP-2 activity in serous effusions will be lower than that measured by zymography, which dissociates MMPs from TIMPs.

DISCUSSION

Our results suggest that the expression profiles of MMP subtypes (i.e., MMP-2, 3, 7, and 9) are specific and depend on the type of effusion. MMP-9 (gelatinase A) activity was prominent in all effusions compared to other MMPs, but its activity was significantly higher in mucous effusions than in serous effusions. This result agrees with a previous report which demonstrated higher activity of both MMP-2 (gelatinase B) and MMP-9 in thick versus thin effusions [10]. However, while differences in the levels of pro-MMP-2 did not show any statistical significance in our data, the active form of MMP-2 was only observed in mucous effusions. Casein zymography also demonstrated that mucous effusions have higher activity of both MMP-3 and MMP-7 than either serous or hemorrhagic effusions, which is the first report of casein zymography being performed on OME effusions. The extracellular matrix, including collagen fibers, is known to support the tympanic membrane and its degradation results in structural changes [16]. The enzymes involved in degrading the extracellular matrix of the tympanic membrane are not well known, however. Increased activity of MMPs has been demonstrated by other investigators in OME fluids and in cholesteatoma tissue [10,12,17]. Furthermore, Antonelli and his colleagues demonstrated significant inhibition of MMP activities by a protease inhibitor in human middle ear effusions [9].

Furthermore, our result showed that TIMP-2 was markedly elevated in the serous effusions, but not in the mucous or hemorrhagic effusions. It is suggested that the extracellular matrix is more actively degraded in the mucous effusion because the mucous effusion is associated with higher activity of MMPs and with saturated levels of endogenous MMP inhibitors (reference to support this claim). MMPs can be inhibited by chelating agents that interact with metal ions, by alpha macroglobulins that entrap MMPs following cleavage of the bait region, and by endogenous tissue inhibitors (TIMPs) [13]. TIMP-2 is unique as a member of the TIMP family, which selectively interacts with MT1-MMP to facilitate the cell-surface activation of pro-MMP-2 and functions as an endogenous inhibitor of angiogenesis [18].

The tympanic membrane maintains unique conical features and proper tension for the sound transmission in varying frequency ranges. Detailed morphological descriptions of the tympanic membrane have been made, and its layers and unique fibrous arrangement are well known [19]. The fibrous layer of the lamina propria provides the tension of the tympanic membrane, and loss of such a layer due to an inflammatory process results in the loss of tension and flaccid membrane [20]. Our preliminary studies failed to show any histological differences between MMP-treated and non-treated tympanic membranes of rats (data not shown). This suggests that MMPs do not act alone in causing structural changes to the tympanic membrane, and that other factors – chronicity, inflammatory mediators, other proteases or pressure effects – are probably involved in this complex process. We hypothesize that MMP activity of middle ear effusions, in combination with other factors, could cause structural damage to the tympanic membrane.

Since Shambaugh first reported two cases with “blue drum membrane” in 1929 [21], there have been many reports of idiopathic blue ear drum or idiopathic hemotympanum[5,7]. The pathogenesis is believed to be the result of negative pressure in the middle ear cavity caused by poor Eustachian tube function in patients with poorly pneumatized mastoid cavity [4,5]. Negative pressure and hypoxia result in mucosal edema and blood vessel rupture. The extravasated blood is broken down to cholesterol and hemosiderin, inducing a foreign body reaction with fibrosis and cholesterol granuloma formation. Although retraction, bulging or

the adhesive nature of the tympanic membrane has been reported in cases with idiopathic hemotympanum with recurrent inflammation [7], the underlying molecular mechanism has not been investigated in detail. Our results demonstrate the activity of MMP-2 and 9 in effusions of idiopathic hemotympanum, which could play a role in conferring structural changes to the tympanic membrane.

In conclusion, we demonstrate structural changes to the tympanic membrane in the OM case and activities of MMP-2, 3, 7 and 9 as well as TIMP-2 in the mucous, serous and hemorrhagic effusions. The data suggest that the expression profile of MMP subtypes and TIMP-2 is specific to effusion type. Further studies are necessary for elucidating its correlation to the natural course or sequelae of OME and idiopathic hemotympanum. The negative findings in our preliminary studies with rat indicate the possible involvement of other factors such as chronicity, inflammatory mediators and proteases, and pressure effects in mediating structural changes in the tympanic membrane in OME.

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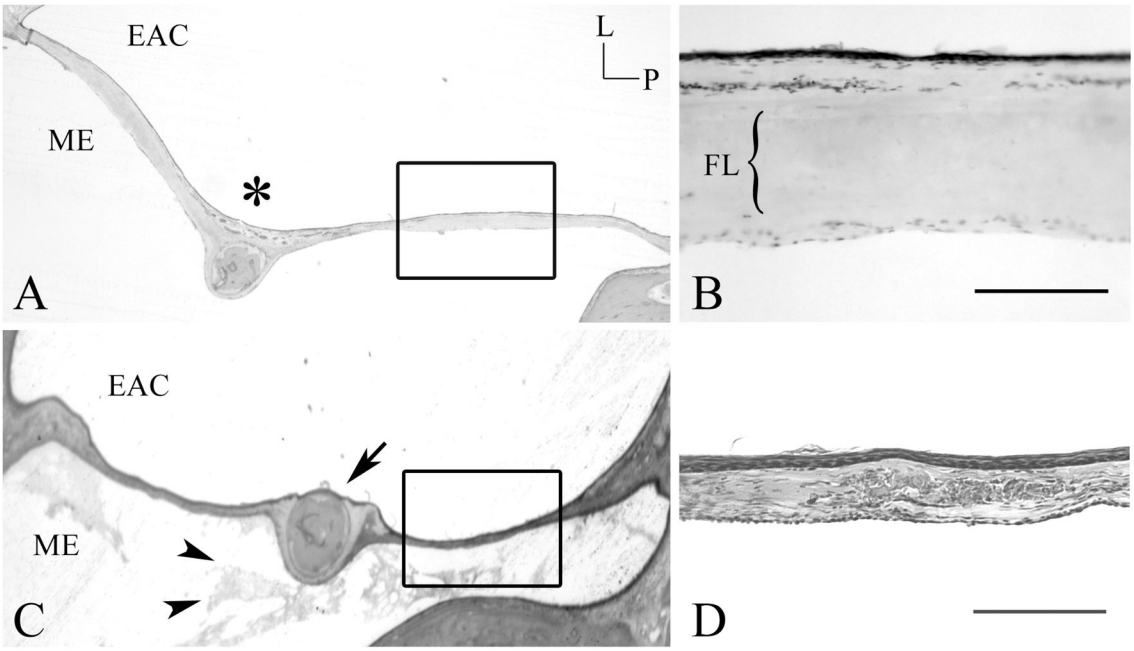

Figure 1.

Archival human temporal bone sections with or without otitis media (OM). Horizontal sections crossing middle of manubrium were stained with hematoxylin and eosin. The tympanic membrane shows a typical conical shape (*) in the normal subject (A) while it is retracted and thinner in the case with OM (C). Blood vessels and connective tissue lateral to manubrium are not seen (arrow) in the case with OM comparing with the normal subject. The fibrous layer (FL) of tympanic membrane is thin and irregular in the case with OM (D), which is intact in the normal subject (B). Arrow heads: middle ear effusion. H & E stain. Original magnification: A and C, x10; B and D, x100. Scale bar: 0.5 mm. EAC: external auditory canal. ME: middle ear. L: lateral side. P: posterior side.

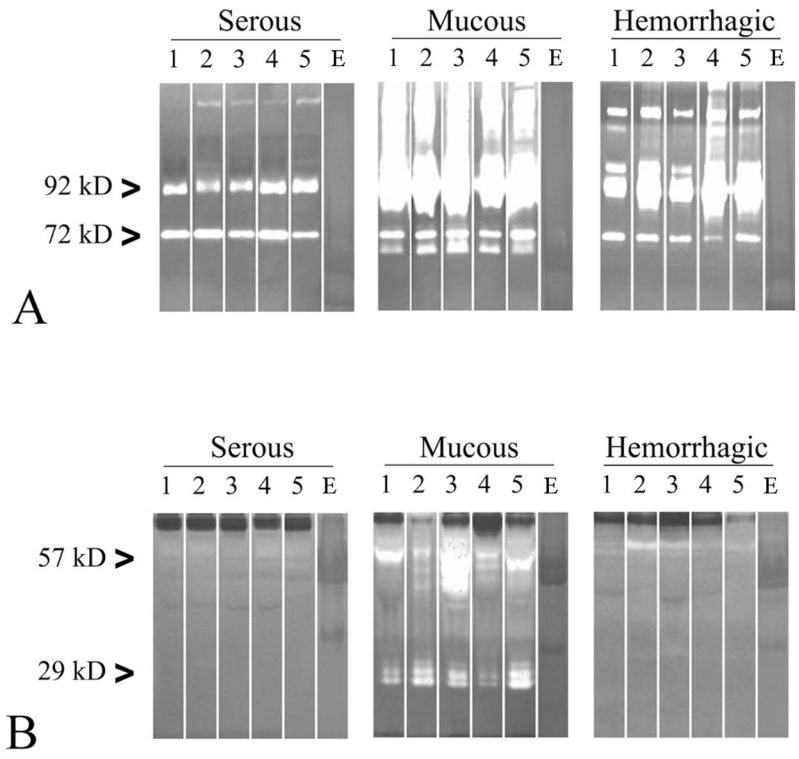

Figure 2.

Gelatin zymography (A) demonstrates MMP-2 (72 kD) activity in all samples. Increased activity of MMP-2 (72 kD) and MMP-9 (92 kD) is present in mucous effusions in comparison with serous and hemorrhagic effusions. The active form of MMP-2 (66 kD) is noted only in mucous effusions, while the active form of MMP-9 (84 kD) is noted in both the mucous and the hemorrhagic effusions. In contrast, casein zymography (B) shows enzyme activities of MMP-3 (29 kD) and MMP-7 (57 kD) only in mucous effusions. EDTA treatment (lane E) completely abloshed these enzyme activities.

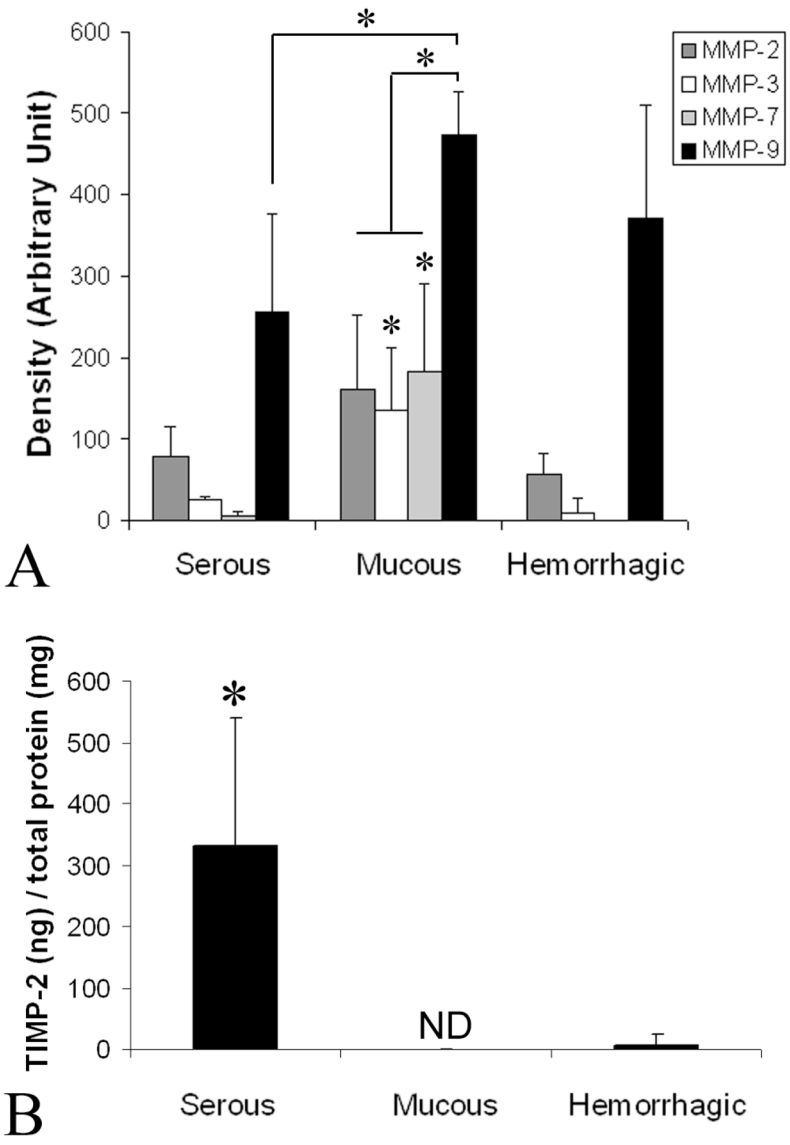

Figure 3.

(A) Denstinometry of zymograms demonstrating different patterns of enzyme activities according to the type of effusion. It is noted that the activity of MMP-9 is highly elevated in all effusions, and is highest in mucous effusions. In contrast, levels of MMP-2 activity were not significantly different. The activities of MMP-3 and 7 are significantly up-regulated in the mucous effusion than in other effusions. (B) TIMP-2 levels according to the type of effusion. It was measured by ELISA, based on a two site sandwich format. The level of TIMP-2 is markedly elevated in serous effusions. Values are given as mean ± standard deviation. ND: not detected. n=5. *: $p<0.05$.

Table 1

Patients profile with effusions of middle ear (N=15).

Effusion	Patient	Age (yr)	Sex	Duration (yr)	PTA (dB)	V-tube History
Serous	1	25	F	7.1	37	1
	2	27	F	2	40	0
	3	42	M	1.2	27	0
	4	38	M	1	35	0
	5	11	M	3.2	37	3
	Mean	28.6		2.9	35.2	0.8
Mucous	1	6	M	3.1	27	1
	2	7	M	3.5	37	2
	3	7	F	1.2	30	0
	4	4	M	0.5	33	0
	5	8	M	3	30	1
	Mean	6.4		2.26	31.4	0.8
Hemorrhagic	1	69	M	0.5	40	0
	2	5	M	1	37	0
	3	4	M	1	30	0
	4	4	M	1.5	35	0
	5	10	F	2.2	40	1
	Mean	18.4		1.24	36.4	0.2

PTA: pure tone average

V-tube: ventilation tube

Table 2

Patients profile of total archival temporal bones, examined in this study.

Group	Age (yr)	Sex	Cause of Death
Control (N = 157)	68.7 ± 15.4	M : 74 F : 83	HA: 28 Malignancy: 26 RP: 10 CVA: 9 Trauma: 6 Others: 10 Unknown: 68
OM History (N = 19)	72.3 ± 12.9	M : 7 F : 12	Malignancy: 2 CVA: 1 HA: 1 Unknown: 15

OM: otitis media, M: male, F: female, CVA: cerebral vascular accident, HA: heart attack, RP: respiratory problem, TM: tympanic membrane

Table 3

Patients profile of the archival temporal bones with/without OM history.

Patient	OM History	Age (yr)	Sex	Cause of Death	Hearing Status	TM thickness (Mean ± SD μm)
#780	No	45	Male	Unknown	Progressive SNHL(B)	690.0 ± 39.6
#393	Yes	55	Female	Unknown	SNHL (B) not related to OM	266.1 ± 13.4*

OM: otitis media, SNHL: sensorineural hearing loss, B: bilateral, TM: tympanic membrane,

*: $p < 0.05$.

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