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MAIN ARTICLE

Serum matrix metalloproteinases and tympanosclerosis

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Abstract

Aim: To investigate levels of matrix metalloproteinases 2 and 9, and of their tissue inhibitor (i.e. tissue inhibitor matrix metalloproteinase 1), in the serum of patients with tympanosclerosis.

Materials and method: We included 40 patients (age range 13–63 years) who had undergone surgery in the ENT department of İzmir Atatürk Training and Research Hospital between 2002 and 2007. Twenty had uncomplicated chronic otitis media and 20 had tympanosclerosis. We also included as the control group 20 individuals with no history of previous otic complaints or systemic or infectious disease. Serum levels of serum matrix metalloproteinases 2 and 9 and of tissue inhibitor matrix metalloproteinase 1 were measured in all subjects and compared.

Result: Significantly higher levels of serum matrix metalloproteinases 2 and 9 were found in the tympanosclerosis group, compared with the chronic otitis media and control groups. There was no statistically significant difference in tissue inhibitor matrix metalloproteinase 1 level between the three groups.

Conclusion: Tympanosclerosis surgery has poor success rates, since the pathological process is still active. We suggest that high levels of matrix metalloproteinases may play a role in the continuation of the disease process.

Key words: Tympanosclerosis; Etiology; Serum; Matrix Metalloproteinase

Introduction

The matrix metalloproteinases are a family of neural endopeptidases which catabolise extracellular matrix components. Their activity is dependent upon calcium and zinc. They act by denaturing collagen in the extracellular matrix, via collagenase (gelatinase) activity, leading to the accumulation of extracellular collagen.[1] Matrix metalloproteinases are released by stromal fibroblasts and inflammatory cells.[2]

Tissue inhibitor matrix metalloproteinase acts to inhibit tissue levels of matrix metalloproteinase. Concentrations of matrix metalloproteinase and tissue inhibitor matrix metalloproteinase are usually balanced to ensure a steady state. However, in some diseases (e.g. atherosclerosis and osteoarthritis) matrix metalloproteinase activity increases.[3] Pathogenetic similarities between atherosclerosis and tympanosclerosis have been described; the pathogenesis of both diseases appears to be related to matrix metalloproteinases.[4] We have previously described a relationship between tympanosclerosis and matrix metalloproteinase 2 (also known as collagenase A), matrix metalloproteinase 9 (also known as collagenase B) and tissue matrix metalloproteinase inhibitor 1.

Materials and methods

The study was approved by the İzmir Atatürk Training and Research Hospital ethics committee.

We included in the study 40 patients who had undergone surgery within the ENT department of İzmir Atatürk Training and Research Hospital between 2002 and 2007. Patients' ages ranged from 13 to 63 years. Twenty patients had uncomplicated chronic otitis media and 20 had tympanosclerosis.

We also included in the study 20 individuals with no history of otic complaints or systemic or infectious disease, as controls.

In the tympanosclerosis group, the male/female ratio was 14/6 and the mean age was 31.8 years (range 16–53). In the chronic otitis media group, the male/female ratio was 12/8 and the mean age 36.9 years (range 13–63). In the control group, the male/female ratio was 10/10 and the mean age 35 years (range 17–61).

Five millilitres of blood was taken from all subjects and stored at −20°C. Levels of matrix metalloproteinase 2, matrix metalloproteinase 9 and tissue inhibitor matrix metalloproteinase 1 were measured using the enzyme-linked immunosorbent assay method.

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Matrix metalloproteinase 2

Serum matrix metalloproteinase 2 concentrations were measured using the Micro-Elisa micro enzyme-linked immunosorbent assay system with Invitrogen solid phase sandwich (Invitrogen, Camarilllo, California, USA). Results were expressed as ng/ml. Standards and samples were pipetted into the pre-prepared wells of the Micro-Elisa system. Any matrix metalloproteinase 2 was bound to immobilising antibodies present in the wells. Wells were washed and biotinylated anti human matrix metalloproteinase 2 antibodies were added. The streptavidin-peroxidase enzyme was then added; this bound to matrix metalloproteinase 2 antibody complexes. Unbound enzymes were removed, and a substrate tetramethylbenzidine solution (TMB) designed to generate a coloured product in the presence of streptavidin-peroxidase was added to the medium. The intensity of colour thus generated was directly proportional to the concentration of matrix metalloproteinase 2 in the sample.

A standard curve of matrix metalloproteinase 2 concentration versus colour intensity was calculated as follows. Standard human matrix metalloproteinase 2 (50 ng/ml), produced with standard diluent buffer, was used to prepare serial dilutions (50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 ng/ml). Following the process described above, colour intensities were measured and plotted against the known matrix metalloproteinase 2 concentration to generate a standard curve.

Serum samples were prepared by diluting 1:15 in standard diluent buffer and mixing well with a vortex mixer. Following the process described above, the colour intensity of each sample was measured. The matrix metalloproteinase 2 concentration was then estimated from the standard curve, and multiplied by 15 to obtain the actual serum concentration.

Matrix metalloproteinase 9

Serum matrix metalloproteinase 9 concentrations were measured using the RayBio® human matrix metalloproteinase 9 enzyme-linked immunosorbent assay system (RayBiotech, Norcross, Georgia, USA). Standard and samples were pipetted into the pre-prepared wells of the system. Any matrix metalloproteinase 9 was bound to the immobilising antibodies present in the wells. Wells were washed and biotinylated anti human matrix metalloproteinase 9 antibodies were added. Unbound biotinylated antibodies were removed by washing, and colour-generating substrate solution TMB (tetramethylbenzidine) was added. The colour intensity generated was proportional to the amount of bound matrix metalloproteinase 9 present. The intensity of generated colour was measured at 450 nm.

A standard curve of matrix metalloproteinase 9 concentration versus colour intensity was calculated using a standard solution of human matrix metalloproteinase 9 (6000 pg/ml) prepared as serial dilutions (6000, 2000, 666.7, 222.2, 74, 24.69, 8.23 and 0 pg/ml). The colour intensities generated by the various matrix metalloproteinase 9 concentrations were measured to generate a standard curve, as for matrix metalloproteinase 2.

The matrix metalloproteinase 9 concentrations in samples were then estimated by comparing each sample's colour intensity to the standard curve.

Tissue inhibitor matrix metalloproteinase 1

The concentration of serum tissue inhibitor matrix metalloproteinase 1 was measured using the RayBio human tissue inhibitor matrix metalloproteinase 1 enzyme-linked immunosorbent assay system. Standard and samples were pipetted into the pre-prepared wells of the system. Any tissue inhibitor matrix metalloproteinase 1 present was bound to the immobilising antibodies present in the wells. Wells were washed and biotinylated anti human tissue inhibitor matrix metalloproteinase 1 antibodies were added. Unbound biotinylated antibodies were removed by washing, and a substrate solution was added which generated colour in proportion to the amount of bound tissue inhibitor matrix metalloproteinase 1 present. The intensity of generated colour was measured at 450 nm.

A standard curve of tissue inhibitor matrix metalloproteinase 1 concentration versus colour intensity was calculated using a standard solution of human tissue inhibitor matrix metalloproteinase 1 (18000 pg/ml) prepared as serial dilutions (18000, 6000, 2000, 666.7, 222.2, 74.07, 24.69 and 0 pg/ml), as for matrix metalloproteinases 2 and 9.

The tissue inhibitor matrix metalloproteinase 1 concentrations in samples were then estimated by comparing each sample's colour intensity to the standard curve.

Statistical analysis

The Windows 16.0 version of the Statistical Package for the Social Sciences software program was used for statistical analysis. One-way analysis of variance was used to compare quantitative data. The post hoc Bonferroni statistical analysis method was used to determine the difference between the groups. A value of $p < 0.05$ was considered statistically significant.

Results and analysis

In the control group, the mean serum matrix metalloproteinase 2 concentration was 78 ng/ml, the mean matrix metalloproteinase 9 concentration 102 ng/ml and the mean tissue inhibitor matrix metalloproteinase 1 concentration 142 ng/ml.

In the chronic otitis media group, the mean serum matrix metalloproteinase 2 concentration was 98 ng/ml, the mean matrix metalloproteinase 9 concentration 109 ng/ml and the mean tissue inhibitor matrix metalloproteinase 1 concentration 149 ng/ml.

In the tympanosclerosis group, the mean serum matrix metalloproteinase 2 concentration was 135 ng/ml, the mean matrix metalloproteinase 9 concentration

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182 ng/ml and the mean tissue inhibitor matrix metalloproteinase 1 concentration 132 ng/ml.

The serum matrix metalloproteinase 2 and 9 levels in the tympanosclerotic group were found to be statistically significantly greater than those in the chronic otitis media group and the control group. There was no statistically significant difference between the tissue inhibitor matrix metalloproteinase 1 levels of the three groups (Tables I and II). There was no statistically significant difference between the matrix metalloproteinase 2 and 9 levels of the control and chronic otitis media groups.

Discussion

Increased matrix metalloproteinase activation results in: the destruction of matrix components within the extracellular compartment; the migration and proliferation of vascular smooth muscle cells; and the growth and proliferation of tumour cells. Such increased matrix metalloproteinase activity is triggered by reactive oxygen radicals, via a reaction with thiol groups within the matrix metalloproteinase.[5] The enzymes xanthine oxidase and (Nadph) nicotinamidadenindinücleotidphosphate oxidase have also been implicated.[6] Recent studies have demonstrated a causative relationship between increased concentrations of free oxygen radicals within the middle ear and the development of myringosclerosis and tympanosclerosis.[7]

In the light of this evidence, the current study aimed to investigate matrix metalloproteinase levels in patients with tympanosclerosis.

Recent research has also demonstrated increased matrix metalloproteinase activity in patients with cholesteatoma and otitis media with effusion.[8] Antonelli *et al.* have shown that matrix metalloproteinase activity is significantly inhibited by some protease inhibitors.[9] It has been suggested that endogenous tissue inhibitors may potentiate this effect by chelating metal ions within the extracellular matrix.[10] Moon *et al.* have suggested that the tympanic membrane changes seen in cases of otitis media with effusion may be related to matrix metalloproteinases; furthermore, these authors have suggested that such changes represent a complex process which also involves other proteases, otic pressure changes and extracellular mediators.[11] Moon *et al.* also analysed samples of effusion fluid taken from these same patients, and found high levels of matrix metalloproteinase 9 in mucous effusions and high levels of tissue inhibitor matrix metalloproteinase 2 in serous effusions.

In the current study, we found no statistically significant difference between the tissue inhibitor matrix metalloproteinase 1 levels of our patient and control groups.

Wilmoth *et al.* exposed the tympanic membranes of 48 Mongolian gerbils to tumour necrosis factor α and bacterial endotoxin *ex vivo*, and then analysed the tympanic membrane matrix metalloproteinase activity. They concluded that the accumulation of these mediators of inflammation within the tympanic membrane extracellular matrix led to increased destruction of the lamina propria, resulting in irreversible pathology (e.g. tympanic membrane retraction, atelectasia and cholesteatoma).[12]

High levels of matrix metalloproteinases and their inhibitors have been found in patients with chronic otitis media, nasal polyposis and Sjögren syndrome.[13] High levels of matrix metalloproteinase 2 and 9 have

TABLE I
MATRIX METALLOPROTEINASE 2 AND 9 AND TISSUE INHIBITOR MATRIX METALLOPROTEINASE 1 CONCENTRATIONS IN THE THREE GROUPS

Group	Mean	SD	Min	Max	*p*
MMP2					
Control	78.27	27.75	29.5	113.7	0.000*
COM	94.63	38.29	28.2	155.7	
TS	135.55	27.81	100.2	191.7	
MMP9					
Control	110.95	56.79	36.3	220.5	0.000*
COM	115.52	28.00	63.3	158.1	
TS	183.18	22.08	147.1	233.6	
TIMP1					
Control	143.14	36.69	62.0	194.0	0.184
COM	157.24	52.20	51.9	274.6	
TS	132.25	36.33	69.7	213.9	

Data represent ng/ml, except for *p* values. *Statistically significant at $p < 0.05$. SD = standard deviation; min = minimum; max = maximum; MMP2 = matrix metalloproteinase 2; COM = chronic otitis media; TS = tympanosclerosis; MMP9 = matrix metalloproteinase 9; TIMP1 = tissue inhibitor matrix metalloproteinase 1

TABLE II
MATRIX METALLOPROTEINASE 2 AND 9 AND TISSUE INHIBITOR MATRIX METALLOPROTEINASE 1 CONCENTRATIONS: COMPARISON OF GROUP MEANS

Grp comparison	Diff btw grp means (ng/ml)	SD (ng/ml)	*p*
TIMP1			
COM *vs* control	14.10	13.41	0.892
COM *vs* TS	24.98	13.41	0.203
Control *vs* COM	−14.10	13.41	0.892
Control *vs* TS	10.89	13.41	1.000
TS *vs* COM	−24.98	13.41	0.203
TS *vs* control	−10.89	13.41	1.000
MMP2			
COM *vs* control	16.36	10.02	0.324
COM *vs* TS	−40.91	10.02	0.000*
Control *vs* COM	−16.36	10.02	0.324
Control *vs* TS	−57.28	10.02	0.000*
TS *vs* COM	40.91	10.02	0.000*
TS *vs* control	57.28	10.02	0.000*
MMP9			
COM *vs* control	4.57	12.24	1.000
COM *vs* TS	−67.66	12.24	0.000*
Control *vs* COM	−4.57	12.24	1.000
Control *vs* TS	−72.23	12.24	0.000*
TS *vs* COM	67.66	12.24	0.000*
TS *vs* control	72.23	12.24	0.000*

*Statistically significant at $p<0.05$. Grp = group; diff btw = difference between; TIMP1 = tissue inhibitor matrix metalloproteinase 1; COM = chronic otitis media; TS = tympanosclerosis; MMP2 = matrix metalloproteinase 2; MMP9 = matrix metalloproteinase 9

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been found in patients with juvenile angiofibroma, a tumour rich in collagen.[14]

In patients with arteriosclerosis (one of the disease processes involving dystrophic calcification), matrix metalloproteinases 2 and 9 have been shown to have elastase activity which results in destruction of the lamellar elastin layer of the arterial wall.[15] Other authors have shown that matrix metalloproteinase 9 causes arterial wall destruction and plaque formation, via increased elastase activity and increased collagen accumulation at the site of arterial wall destruction.[16,17]

We found high levels of matrix metalloproteinase 9 in our patients with tympanosclerosis, suggesting a similar involvement of this enzyme in the development of tympanosclerotic plaques.

Yasmin *et al.* found high levels of matrix metalloproteinases 2 and 9 in the serum of patients with isolated systolic hypertension due to atherosclerosis (mean matrix metalloproteinase 2 levels were 177 ng/ml in patients and 151 ng/ml in controls, while mean matrix metalloproteinase 9 levels were 157 ng/ml in patients and 119 ng/ml in controls).[18] In our study, the difference in matrix metalloproteinase levels between the patient groups and the control group was similarly statistically significant.

The relationship between tympanosclerosis and atherosclerosis has been investigated, since their pathogenesis is similar. Koç *et al.* found myringosclerosis in 66.6 per cent of 1024 atherosclerotic patients who underwent otoscopic inspection, and suggested that there may be a genetic predisposition linking both conditions.[19]

Another disease process involving dystrophic calcification is osteoarthritis. Increased concentrations of free oxygen radicals and some cytokines have been found in patients with osteoarthritis, and steroid injections have been used as treatment.[20] Tetracyclines, which chelate calcium ions and inhibit tissue metalloproteinases, have also been used for osteoarthritis. Tetracyclines have previously been shown *in vitro* to be potent anti-calcification agents in tissues with ectopic calcification.[21] Doxycycline has been found to reduce the severity of osteoarthritis in dogs.[22] Doxycycline and tetracycline are used as antibiotics, but are also non-selective matrix metalloproteinase inhibitors. Axisa *et al.* showed that doxycycline accumulates in atherosclerotic plaques and prevents plaque progression and rupture by inhibiting matrix metalloproteinase 1 within the plaque.[23] Ozcan *et al.* investigated acute otitis media in 25 adult guinea pigs after myringotomy; the animals' right ears were treated with topical doxycycline while their left ears were left as controls.[24] At the end of the sixth week, histological evidence of myringosclerosis was found in significantly less of the ears treated with doxycycline. These findings support our own discovery of high levels of matrix metalloproteinases in patients with tympanosclerosis.

Topical applications of ascorbic acid, N-acetylcystein and vitamin E have all been used in an attempt to prevent myringosclerosis; all these agents are reported to act by reducing the concentration of reactive oxygen radicals.[25] We can say this agents decrease the oxigen free radicals so matrix metalloproteinase levels and decrease myringosclerosis.

- **Surgical treatment of tympanosclerosis has a poor success rate, as the primary pathological process is still active**
- **This study found significantly higher matrix metalloproteinase concentrations in tympanosclerotic patients, compared with normal controls and patients with chronic otitis media**
- **Matrix metalloproteinase inhibitors have begun to be used to restrict the spread of some cancer types, and may also be useful in the treatment of tympanosclerosis**

Other methods of inhibiting matrix metalloproteinase activity have been reported, such as the use of angiotensin-converting enzyme inhibitors following acute myocardial infarction.[26] In this clinical context, these drugs are proposed to balance the concentrations of matrix metalloproteinase 1 and tissue inhibitor matrix metalloproteinase 1 (MMP1).[26]

Since matrix metalloproteinases also promote tumour invasion and progression, it has been postulated that tissue matrix metalloproteinase inhibitors may be useful during the follow up of most types of cancer, to prevent metastases and relapses. High concentrations of matrix metalloproteinases have been found in many cancer types (e.g. prostate, pancreas, lung, breast and colon) and are considered to indicate a poor prognosis.[27] Synthetic matrix metalloproteinase inhibitors have recently begun to be used in cancer treatment; preparations include Batimastat (British Biotec, Oxford, UK), which inhibits matrix metalloproteinases 1, 2, 3 and 9, and Marimastat, its second generation derivative.[28]

We suggest that the use of matrix metalloproteinase inhibitors in atherosclerosis, osteoarthritis and tympanosclerosis may reduce matrix metalloproteinase concentrations and thereby retard and attenuate the pathological mechanism of disease.

Conclusion

The main treatment in tympanosclerosis is surgery. Because the process continues to be active. Therefore the medical treatment methods are still being investigated. We think that using synthetic matrix metalloproteinase inhibitors in the future will be efficient in such kinds of these patients.

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