ORIGINAL RESEARCH

Potential role of matrix metalloproteinase-2,-9 and tissue inhibitors of metalloproteinase-1,-2 in exudative pleural effusions

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Abstract

Purpose: To investigate diagnostic values of pleural fluid matrix metalloproteinase-2 (MMP-2), MMP-9, tissue inhibitors of metalloproteinase-1 (TIMP-1) and TIMP-2 measurements in tuberculous pleurisy (TP) and malignant pleurisy (MP).

Methods: The study included 24 patients with TP, 22 patients with MP and 15 patients with pleural effusion of non-tuberculous and non-malignant origin as controls. MMP-2,-9 and TIMP-1,-2 levels in pleural fluid were measured by ELISA method.

Results: Pleural fluid MMP-2 and MMP-9 levels were higher ($P<0.001$, $P<0.001$, respectively) in TP than in MP and controls. MP patients have higher pleural fluid MMP-2 and MMP-9 levels ($P<0.01$, $P<0.05$, respectively) than controls. Pleural fluid TIMP-2 levels were higher ($P<0.01$ and $P<0.001$, respectively) in MP than in TP and controls. Pleural fluid MMP-9 levels were negatively correlated with pleural fluid TIMP-2 levels ($r: 0.464$, $P=0.029$) in patients with MP.

Conclusions: Determination of TIMP-2 in pleural fluid may contribute to differentiate TP from MP. These results suggest that overproduction of MMP-9 and TIMP-2 is associated with accumulation of the pleural effusion in ma-

lignancy. Further studies with a greater number of patients are needed to confirm this hypothesis.

The accumulation of fluid in the pleural space indicates the presence of systemic or local disease.¹ Tuberculosis and cancer are the main causes of pleural exudates. The pathophysiology of malignant effusions is multifactorial and is incompletely understood. Because no serious complications occur, the most accurate option for the diagnosis of pleural effusion is a pleural biopsy. Malignant pleural effusions (MPEs), which comprise a heterogeneous group of conditions, represent an important source of morbidity for patients with underlying cancer. They can occur as the initial presentation of cancer, as a delayed complication in patients with previously diagnosed malignancies, or as the first manifestation of cancer recurrence after therapy. Detection of an effusion coincident with a newly diagnosed cancer does not establish an MPE because 50% of such effusions are nonmalignant.²

Malignant and tuberculous pleural effusions are lymphocytic pleural effusions.^{3,4} One of the major issues in differential diagnosis of tuberculous pleural effusion (TPE) is its early recognition and differentiation from MPE. Early treatment of TPE is curative and decreases the possibility of complications, while early treatment of MPE may increase quality of life and survival of patients with advanced malignant disease.⁵⁻⁹ TPE is still difficult to diagnose, especially in regions with a low incidence of tuberculosis. The combination of different invasive and noninvasive techniques in the diagnosis of TPE may provide optimal results.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases, which together have the capacity to breakdown all components of the extracellular matrix.¹⁰ MMPs are of considerable biomedical interest because they have been implicated in many pathological processes characterized by dysregulated turnover of connective tissue matrices, such as occurs in rheumatoid and osteoarthritis, periodontal disease, metastatic cancer, metabolic bone disease, aortic aneurysm and atherosclerosis, sterile corneal ulceration, dystrophic epidermolysis bullosa, lung damage associated with chronic obstructive pulmonary disease, and emphysema.¹⁰⁻¹⁴ Because of their powerful degradative capacity, the activity of MMPs is tightly regulated by a family of tissue inhibitors of metalloproteinases (TIMPs), as well as other proteinase inhibitors, such as α 2-macroglobulin and tissue factor pathway inhibitor-2.¹⁰

The raised expression in MMP isoforms, associated with some pulmonary diseases (cystic fibrosis, emphysema, bronchiectasis, adult respiratory distress syndrome, lung cancer),¹⁵ is responsible for the degradation and disruption of the ECM structure and emphasizes the biological significance of these enzymes in the lung.¹⁶ The proteolytic balance between MMPs and TIMPs is important in normal tissue remodeling and various pathological conditions.

MMP-2 and MMP-9 form a gelatinase subgroup of MMPs and both of these MMPs can be used to dis-

tinguish between infectious and neoplastic processes. Therefore, we aimed to investigate the role of pleural fluid MMP-2, MMP-9, TIMP-1 and TIMP-2 measurements in TP and MP and also to evaluate the association between these enzymes and type of pleural effusion.

Materials & Methods

The study population

Samples utilized in this study were provided from Istanbul Medical Faculty, Department of Internal Medicine and Yedikule Chest Disease and Chest Surgery Education and Research Hospital, İstanbul, Turkey. Twenty four patients (M/F-16/8, mean age 27.1 ± 12.2 yr) with TP, 22 patients (M/F-13/9, mean age 55.2 ± 10.1 yr) with MP (10 squamous cell lung cancer, 6 lung adenocarcinoma, 2 malignant mesothelioma, 1 thymoma, 2 stomach adenocarcinoma, 1 acute myeloblastic leukemia) and 15 patients (M/F-7/8, mean age 46.3 ± 10.9 yr) with pleural effusions of non-tuberculous and non-malignant origin as controls (2 congestive heart failure, 2 empyema, 3 pulmonary thromboembolism, 8 non-specific pleuritis) with no previous treatment were included in the study. Normally, very small amounts of pleural fluid are present in the pleural space, and fluid is not detectable by routine methods. When certain disorders occur, excessive pleural fluid may accumulate and cause pulmonary signs and symptoms. Patients with pleural effusion of non-tuberculous and non-malignant origin were included in the study as controls because thoracentesis cannot be performed in healthy individuals.

Pleural effusion was, at first, collected for diagnostic or therapeutic purposes (to decrease the respiratory problems). In these patients thoracentesis was indicated and they underwent collection of pleural fluid after they signed an informed consent form. After diagnosis, some of the fluids were used for the study. All samples were designed as exudates according to Light's criteria.¹⁷ The fluid was defined as exu-

dates if it fulfilled at least one of the following criteria: pleural/serum ratio of total protein greater than 0.5; pleural/serum ratio of total lactate dehydrogenase > 0.6; or pleural lactate dehydrogenase (LDH) > two-thirds of upper limit of normal for serum LDH (>400 IU/L). The effusions were moderate in size, unilateral and predominantly lymphocytic. Diagnosis of tuberculosis was based on compatible clinical and radiological signs associated with a *M. tuberculosis*-positive culture from fluid or pleural fragments, the presence of caseous granuloma in the pleura, or both. The presence of tumour cells in the pleural fluid or tissue indicated cancer. Malignant pleural effusion was diagnosed by pleural effusion cytology. Diagnoses in the control group were made according to standard clinical and laboratory evaluations.

Exclusion criteria

Patients with complications such as renal, endocrine or hepatic diseases, diabetes mellitus, obesity, viral and other bacterial infections etc were excluded from the study. Patients, not yet placed on medication, were used since most antibiotics used for treatment of patients may modify levels of parameters.

Specimen Collection and Processing

Thoracentesis was performed in the usual manner, and the pleural tissue samples were obtained by percutaneous needle biopsy, except four, which were biopsied by thoracoscopy. The effusions were generally moderate in size and unilateral in both groups. All specimens were received as fresh effusions. A portion of the pleural effusion sample was submitted for acid-fast staining, cytological examination and measurement of pH, protein, albumin, lactate dehydrogenase (LDH) and glucose. Total cell, white cell and differential cell counts (Giemsa stain) were obtained by counting at least 200 cells under a light microscope. Pleural fluid samples were collected in dry tubes. Samples were brought immediately to the laboratory, centrifuged for 10 min at 1500 g, then stored at -80°C. All parameters

were analyzed in all samples together in a single batch, after completion of the protocol (control and patient samples were analysed in the same batch).

Biochemical analysis

Assays were performed following the manufacturer's recommendations. All sample concentrations were measured in duplicate at the beginning and end of each run.

Assay of pleural effusion MMP-2 Concentrations:

Pleural effusion MMP-2 levels were determined by ELISA (RayBiotech, Inc. US., Human MMP-2 ELISA Kit, Cat#: ELH-MMP2-001). The minimum detectable dose of MMP-2 is typically < 3.5 ng/ml. Intra- and interassay coefficients of variation for MMP-2 were 10 % and 11.9%, respectively.

Assay of pleural effusion MMP-9 Concentrations:

Pleural effusion MMP-9 levels were determined by ELISA (RayBiotech, Inc. US., Human MMP-9 ELISA Kit, Cat#: ELH-MMP9-001). The minimum detectable dose of MMP-9 is typically < 10 pg/ml. Intra- and interassay coefficients of variation for MMP-9 were 10 % and 11.8%, respectively.

Assay of pleural effusion TIMP-1 Concentrations:

Pleural effusion TIMP-1 levels were determined by ELISA (RayBiotech, Inc. US., Human TIMP-1 ELISA Kit, Cat#: ELH-TIMP1-001). The minimum detectable dose of TIMP-1 is < 40 pg/ml. Intra- and interassay coefficients of variation for TIMP-1 were 9.8% and 10.9%, respectively.

Assay of pleural effusion TIMP-2 Concentrations:

Pleural effusion TIMP-2 levels were determined by ELISA (RayBiotech, Inc. US., Human TIMP-2 ELISA Kit, Cat#: ELH-TIMP2-001). The minimum detectable dose of TIMP-2 is typically less than 10 pg/ml.

TABLE 1. Parameters used to determine metalloproteinases and tissue inhibitors of metalloproteinases of pleural effusions of all patients.

	Non-tuberculous/ non-malignant n=15 (M/F=7/8)	Tuberculous n=24 (M/F=16/8)	Malignant n=22 (M/F=13/9)
MMP-2(ng/ml)	887.1±259.25	2230.2±316.5 ^{a*}	1779.6±262.7 ^{a*, b*}
MMP-9 (ng/ml)	6.0±1.5	49.5±14.9 ^{a*}	17.2±7.0 ^{***, b*}
TIMP-1 (ng/ml)	888.4±247.2	2499.3±352.3 ^{a*}	2126.6±406.9 ^{a*, b**}
TIMP-2 (ng/ml)	74.4±16.8	87.±21.5	113.7±26.3 ^{a*, b**}
Protein (ng/ml)	3973.3±518.8	5079.2±584.6 ^{a*}	4318.2±561.2 ^{b*}

MMP-2: Matrix metalloproteinase-2; MMP-9: Matrix metalloproteinase-9; TIMP-1: Tissue inhibitors of metalloproteinase-1; TIMP-2: Tissue inhibitors of metalloproteinase-2

a; statistically different from pleurisy originated non-tuberculous/ non-malignant

b; statistically different from pleurisy originated tuberculous

* $P<0.001$; ** $P<0.01$

Intra- and interassay coefficients of variation for TIMP-2 were 10% and 12%, respectively.

Statistical analysis

Demographic data were expressed as mean±SEM. Data are presented as the mean ± SEM. Kruskal–Wallis analysis of variance test was used to examine significant intergroup differences and if significant, the Mann–Whitney U test was used for between-group comparisons. Correlations analysis was tested using Pearson's or Sperman's correlation. Differences were considered statistically significant when $P<0.05$. Data were analyzed by statistical software (SPSS for Windows 10.0; SPSS, Chicago, IL).

Results

Pleural fluid MMPs, TIMPs and protein concentrations in the study are shown in Table 1.

Pleural fluid MMP-2 and MMP-9 levels were higher ($P<0.001$, $P<0.001$, respectively) in TP than in MP and controls. MP patients had higher pleural fluid MMP-2 and MMP-9 levels ($P<0.01$, $P<0.05$, respectively) than controls. Pleural fluid TIMP-1 levels were higher ($P<0.01$, $P<0.001$, respectively) in TP than in MP and controls. MP patients had higher pleural fluid TIMP-1 levels ($P<0.001$) than controls. Pleural fluid TIMP-2 levels were higher ($P<0.01$ and $P<0.001$, respectively) in MP than in TP and controls. There was no difference in pleural fluid TIMP-2 levels between

TP and control patients. Pleural fluid protein levels were higher ($P<0.001$, $P<0.001$, respectively) in TP than in MP and controls. There was no difference in pleural fluid protein levels between MP and controls patients.

Since protein levels in TP were much higher than in other groups, intergroup comparisons were performed using levels adjusted with respect to pleural fluid protein concentrations, as well as with absolute values (Table 2). When MMP-2 levels were adjusted with respect to pleural fluid total protein concentrations. The ratios (MMP-2/total protein) were lower in controls than in TP and in MP ($P<0.001$). There was no difference in this ratio between TP and MP. The ratio (MMP-9x10⁻²/total protein) was higher in TP than in MP and in controls ($P<0.001$) but MP patients had higher ratios (MMP-9x10⁻²/total protein) than controls ($P<0.01$). The ratio (TIMP-1/total protein) was lower in controls than in TP and in MP ($P<0.001$). There was no difference in this ratio between TP and MP nor was there a difference in the ratio (TIMP-2/total protein) between TP and controls, whereas the ratio in MP was higher than in the other two groups ($P<0.01$).

Correlations analyses

Correlations between metalloproteinases, tissue inhibitors of metalloproteinases and protein in pleural effusions of patients are depicted in Figure 1.

TABLE 2. Ratios between metalloproteinases, tissue inhibitors of metalloproteinases and protein in pleural effusions of patients.

	Non-tuberculous/ non-malignant n=15 (M/F=7/8)	Tuberculous n=24 (M/F=16/8)	Malignant n=22 (M/F=13/9)
MMP-2/TP	0.2±0.1	0.5±0.1 ^{a*}	0.4±0.1 ^{a*}
MMP-9x10 ⁻² /TP	1.6±0.5	9.9±3.2 ^{a*}	4.0±1.7 ^{a*, b**}
TIMP-1/TP	225.5±62.7	499.6±97.1 ^{a*}	500.9±113.0 ^{a*}
TIMP-2/TP	18.7±3.4	17.9±3.6	26.9±7.5 ^{a**, b**}

MMP-2: Matrix metalloproteinase-2; MMP-9: Matrix metalloproteinase-9; TIMP-1: Tissue inhibitors of metalloproteinase-1; TIMP-2: Tissue inhibitors of metalloproteinase-2; TP: Total protein.

a; statistically difference from pleurisy originated non-tuberculous/ non-malignant

b; statistically difference from pleurisy originated tuberculous

* $P<0.001$; ** $P<0.01$.

Correlation analysis in patients with TP: Pleural fluid MMP-2 levels were correlated with pleural fluid TIMP-2 levels ($r: 0.485$, $P=0.016$), and pleural fluid MMP-2 levels were negatively correlated with pleural fluid TIMP-1 levels ($r: -0.441$, $P=0.031$).

Correlation analysis in patients with MP: Pleural fluid MMP-9 levels were negatively correlated with pleural fluid TIMP-2 levels ($r: 0.464$, $P=0.029$).

Correlation analysis in patients with controls: A correlation was found between TIMP-2 and protein ($r: 0.641$, $P=0.010$).

There was no correlation among other parameters in the groups.

Discussion

Diagnosing the etiology of pleural lymphocytic exudate is a challenging medical problem because of a lack accuracy of non invasive investigations.¹⁸⁻²³ Identifying new biochemical fluid markers is therefore suitable for differentiating benign from malignant lesions. We evaluated the role of MMPs and TIMPs in PE pathogenesis. TIMP-2 concentration were highest in MP. MMP-9 showed a negative correlation with TIMP-2 in MP. Moreover, there was no difference in the ratio (TIMP-2/total protein) between TP and controls, whereas the ratio in MP was higher than in the other two groups. One possible scenario, which explains the role of elevated TIMP-2 in advanced cancer, is that increased expression of TIMP-2 could be related to the increase of MMP expression during tu-

mour progression. As supported in previous studies, there was a relationship between TIMP-2 and MMPs.²⁴⁻²⁶ TIMP-2 produced by tumor cells is an important determinant of their capacity to induce the formation of MPE and may be a useful target for the treatment of malignant pleural disease.

Although lymphocytic pleural effusions are common in exudative pleural effusions due to malignancy, MMP-2, MMP-9, TIMP-1 concentrations, in our study, were highest in TP and were unexpectedly higher than in MP and non-tuberculous and non-malignant pleural effusions. Malignant pleural effusions, excluding malignant mesothelioma, had low cell component concentrations because the pleura had only recently been affected by the tumour. We believe that this could explain the lower MMP-2, MMP-9, TIMP-1 concentrations in pleural effusions due to malignancy. Pleural fluid MMP-2 levels were also correlated with pleural fluid TIMP-2 levels, and pleural fluid MMP-2 levels were negatively correlated with pleural fluid TIMP-1 levels. Although total protein levels were the highest in TP, the ratio (MMP-9x10⁻²/total protein) was higher in TP than in MP and in controls. The pleural effusion of tuberculosis caused by reactive response of lymphocytes is associated with increased pleural permeability and local interaction of cells and cytokines, which possibly contribute to MMP-9 accumulation.^{27,28} Jin et al.²⁷ showed that the levels of MMP-9 are high in the pleural fluids of patients with tuberculosis and even higher in the patients with lung cancer compared with the patients

FIGURE 1. Correlations between metalloproteinases, tissue inhibitors of metalloproteinases and protein in pleural effusions of patients: (A) and (B) in TP, (C) in MP and (D) in pleurisy originated non-tuberculous/ non-malignant.

MMP-2: Matrix metalloproteinase-2; MMP-9: Matrix metalloproteinase-9; TIMP-1: Tissue inhibitors of metalloproteinase-1; TIMP-2: Tissue inhibitors of metalloproteinase-2; TP: Total protein.

with liver cirrhosis. The pleural effusion in tuberculosis caused by the reactive response of lymphocytes is associated with increased pleural permeability and local interaction of cells and cytokines, which possibly contribute to MMP-9 accumulation.²⁸ In this study, they showed the correlation between the num-

ber of lymphocytes and the levels of MMP-9 in the pleural fluids of patients with liver cirrhosis, tuberculosis, and lung cancer. MMP-9 produced by lymphocytes may be present in the pleural fluids of patients with tuberculosis. The clinical use of MMP-9 measurement in the pleural effusion as a differential diag-

nostic parameter or diagnostic reference may be possible with other parameters in their study.

As our results demonstrated, deregulation of the MMP-TIMP balance can result in the progression of many human diseases, including cancer.²⁹⁻³¹ In this limited study, we have confirmed that proteolytic processes within the pleural space may alter the integrity of the mesothelial cell layer and/or the underlying basement membrane and, therefore, facilitate fluid influx into the pleural space patients with MP and TP.³² Moreover, in patients with pleural effusion of non-tuberculous and non-malign origin, TIMP-2 levels were related to the increased protein levels.

These results suggest that overproduction of MMP-9 and TIMP-2 is associated with accumulation of the pleural effusion in malignancy. The potential role of MMP-9 as a possible sensitive molecular marker implicated in the infectious and neoplastic processes, which could in part be responsible for the proteolytic activities in pleural effusions. In conclusion, in the differential diagnosis of pleural effusions higher MMP-2,-9 and TIMP-1,-2 levels may prove to be a rapid, inexpensive, practical and accurate method of differentiating one from the other exudate types. In particular, pleural MMP-2 levels may also be helpful in discriminating malignant from non-malignant pleural effusions. Further studies with a greater number of patients are needed to confirm this hypothesis.

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