Pleural fluids associated with metastatic lung tumors are rich in progelatinase B/proMMP-9*

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Gelatinase B/MMP-9 is a member of matrix metalloproteinases with a major role in extracellular matrix degradation, cell proliferation and migration. Its proenzyme form has also been reported in pleural fluids as an inducible species, but its relation to pleural pathology has not yet been fully clarified. The primary goal of this study was to evaluate proMMP-9 as a potential marker for differentiating pleural effusions of both malignant and non-malignant origin.

Pleural fluid samples were studied from 194 patients, including tumor etiology in 133 cases, inflammatory disorders in 33, transudates in 12, and unspecified disorders in 16 patients. The concentrations of proMMP-9 were estimated by means of immunoassays and/or by scanning zymography. Samples were also examined for C-reactive protein (CRP).

The analysis of proMMP-9 showed significant differences among the etiological groups with the highest concentrations in parainflammatory exudates, intermediate in paraneoplastic exudates, and the lowest in transudates. However, the analysis of the paraneoplastic group revealed a distinct heterogeneity with a minor portion of fluids reaching values typical for parainflammatory effusions. A subsequent sorting based on tumor histology showed increased levels particularly in exudates associated with metastatic tumors. Interestingly, proMMP-9 values in general correlated with CRP, a systemic marker of inflammation.

Thus, MMP-9 proenzyme appears to complement traditional markers distinguishing pleural fluids of different origin. Yet, the differentiation between paraneoplastic and parainflammatory exudates must be regarded with caution due to the presence of a high-expressive paraneoplastic subpopulation, including effusions associated with metastatic tumors.

Key words: MMP-9, pleural effusion, lung cancer, metastasis, C-reactive protein

Gelatinases make up a distinct mechanistic group of metalloproteinases (MMPs) with major role in the remodeling of extracellular matrix components, cell proliferation and migration. Proenzyme forms of gelatinases have been detected in substantial amounts in pleural fluids of different origin [13]. Whereas gelatinase A (MMP-2), similarly to interstitial collagenase (MMP-1), seems to be expressed constitutively, the expression of gelatinase B (MMP-9) is inducible under pathological conditions [8]. Therefore, MMP-9 has been considered among potential indicators for the differentiation of pleural fluids.

However, the reports on the degree of expression of MMP-9 among clinically relevant effusion types have so far been contradictory. The first study found the expression ratio of gelatinase B to gelatinase A highest in exudates of paraneoplastic origin [8], whereas other reports showed higher mean values in parainfectious exudates as compared to paraneoplastic exudates and transudates [9, 17]. The former observation has been supported by the findings of elevated MMP-9 expression in both cancer and stroma cells [5, 24], the latter by the reports demonstrating proMMP-9 secretion by inflammatory cells in areas of inflammation [12, 18, 21, 22, 25, 27, 30, 31]. ProMMP-9 has in fact been identified as a secretion product of macrophages, T cells and granulocytes and found in specific granules of these cells [3]. In addition, recent studies have also demonstrated systemic MMP-9 upregulation in a variety of diseased states, such as

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cancer, inflammatory diseases, and even under stress conditions such as trauma and surgery [6, 7, 20], indicating a possible MMP-9 role in host-response.

Therefore, the primary goal of the present study was to explore in an extended study group the expression of MMP-9 in clinically relevant effusion types, considering histology-based composition of the paraneoplastic effusion group, and to evaluate proMMP-9 as a potential marker in differential diagnosis of pleural effusions. Furthermore, to address a posssible relationship between MMP-9 expression and the host-response reaction, a comparison of pleural proMMP-9 expression with both pleural and circulating levels of a model acute-phase reactant, the C-reactive protein was included.

We found significant differences in the expression of MMP-9 among transudates, parainflammatory effusions and paraneoplastic effusions. However, a more detailed analysis of the tumor-associated group revealed a minor subpopulation which was rich in metastatic tumors and showed increased pleural proMMP-9 production. ProMMP-9 values correlated with both local and systemic levels of CRP.

Patients and methods

Patients. Pleural effusion samples were obtained from 194 patients undergoing thoracentesis/thoracoscopy for therapeutic/diagnostic reasons. Pleural fluids were sorted into four groups: paraneoplastic effusions (n=133), parainflammatory effusions (33), transudates (12), and effusions of unspecified etiology (16). The diagnoses were based on clinical data, biopsy, cytologic examination of the pleural fluid and usual biochemical tests. The study group consisted of 121 males and 73 females. The mean age was 63 years ±11 (S.D.). A subgrouping of oncological patients according to histology showed prevalence of adenocarcinomas (n=42), followed by squamous carcinoma (17), mesothelioma (11), and small-cell carcinoma (9). Remaining minor histologic types were pooled separately (28). Metastatic tumors (n=26) included tumors of renal origin (6), breast tumors (5), gastrointestinal tumors (4), ovarian tumors (3), uterovulvar tumors (3), osteal tumors (2), and metastases of unknown primary site (3). Majority of tumors were classified in Stage IV (58%), IIIB (28%), IIIA (4%), IB-II(3%), in 7% the staging was unspecified/not verified. Most patients were treated by chemotherapy and actinotherapy in the past but the therapy was completed by one month before the examination of pleural fluid. Within one month before the examination about 18% patients were treated mostly by chemotherapy or biological/hormonal therapy, or were not treated yet. The underlying etiology in parainflammatory group were bronchopneumonias except for two tuberculosis effusions. Transudates were mostly caused by a congestive heart failure, one effusion was induced by malignancy in the abdominal cavity. All patients gave their informed consent and the protocol was approved by the University Hospital Ethical Committee.

Methods. The fluid supernatants of pleural effusions (centrifuged at 170 g, 10 min, RT) were placed into heparin-coated tubes to reduce fibrin depositions and either immediately subjected to laboratory analysis, or leaving aliquots stored at –70 °C until used for further analyses.

The concentrations of proMMP-9 were estimated by means of immunoassays and/or by scanning substrate electrophoresis using quantitative standards. MMP-9 ELISA sandwich system (Amersham Pharmacia Biotech) was employed according to the recommendation of the manufacturer, in doublets. This system detects proMMP-9 and proMMP-9/TIMP-1 complex but no active MMP-9. Gelatinolytic activities of samples were determined through gelatin zymography according to KLEINER and STETLER-STEVENSON [16]. In brief, aliquots (10 μl) were applied to a denaturing 8% SDS-polyacrylamide gel containing 0.15% gelatin. Following electrophoresis, gels were washed for 1 h in 2.5% Triton X-100 to remove SDS, and incubated in an enzyme buffer containing 50 mM Tris-HCl (pH 7.3), 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35 for 18 h at 37 °C. Bands of enzymatic activity were vizualized by negative staining with Coomassie brilliant blue dye solution. Stained gels were soaked with 1% glycerol and dried between two sheets of nitrocellulose film. Zymograms were scanned on a Umax UTC-2100 transparency scanner and quantified by means of the SigmaGel (SPSS Science) densitometric program using purified human proMMP-9 (APBiotech), proMMP-9 band in MMP-Control-1 media (Sigma), and previous, immunometrically analyzed specimens, as quantitative standards. In cases where both quantitative methods were employed, immunometric assays only were used for subsequent statistical analyses. The substrate for zymography was gelatin purchased from Bio-Rad Laboratories, all other analytical grade reagents were purchased from Sigma-Aldrich.

Pleural fluid samples were examined for total protein, C-reactive protein (CRP), and lactate dehydrogenase (LDH) activity by using standard laboratory methods in the Department of Clinical Biochemistry, University Hospital in Pilsen. To compare the intrapleural and circulating CRP values, available blood sera were also analyzed for CRP.

Statistical analyses. Analytical data were evaluated primarily by non-parametric tests, such as a Kruskal Wallis test, Wilcoxon test, and Spearman rank correlation.

Results

Expression of MMP-9 in pleural fluids of different origin. As a measure of MMP-9 expression, the MMP-9 ELISA sandwich system and the substrate SDS-PAGE were employed, as described in detail in section Methods. Figure 1 shows a typical activity pattern of both gelatinases in paraneoplastic effusions, parainflammatory effusions, and transudates, obtained by the substrate SDS-PAGE zymography. It should be noted that under the conditions of an zymographic assay, proMMP-9 becomes proteolytically ac-

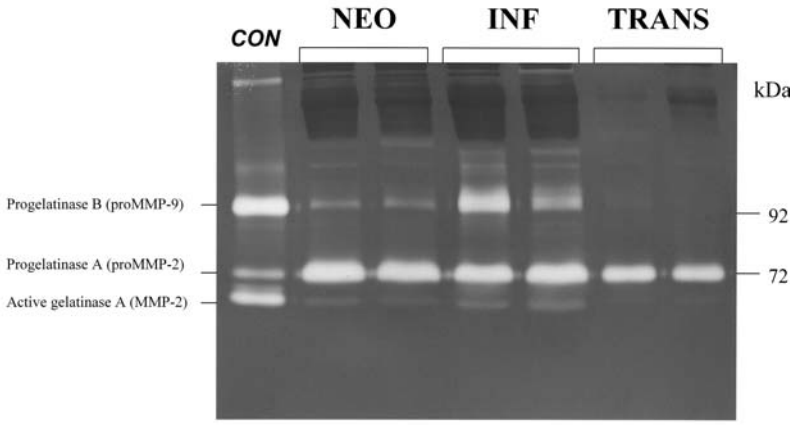

Figure 1. Gelatin zymography of representative paraneoplastic exudates (NEO), representative parainflammatory exudates (INF), and representative transudates (TRANS). Individual groups exhibit differential expression of progelatinase B (MMP-9), as revealed by intensities of gelatinolytic bands (see Methods). The first lane (CON) contains a positive control from medium of activated human fibroblasts (MMP Control-1, Sigma).

Table 1. Progelatinase B/proMMP-9 concentrations in pleural effusions of different origin

Effusion	Paraneoplastic[1]	Parainflammatory[2]	Transudate
Mean	34.4 ng/ml	137 ng/ml	2.9 ng/ml
Interquartile range	4, 30	20, 112	0.6, 5
Median	8.4 ng/ml	49.7 ng/ml	1.5 ng/ml
n	133	33	12

[1]Statistically significant to parainflammatory effusions at $p<0.001$ and to transudates at $p<0.01$; [2]Statistically significant to transudates at $p<0.001$

Table 2. Progelatinase B/MMP-9 concentrations in paraneoplastic effusions associated with tumors of different histology

Malignancy		n	Progelatinase B/MMP-9 Median value (ng/mL)	Interquartile range	Statistical significance (Wilcoxon test)
Metastatic tumors		26	34.2	12.0, 61.0	$p < 0.01$[1]
Primary Lung Tumors	Adenocarcinoma	42	8.2	4.6, 17.5	$p < 0.01$[2]
	Squamous carcinoma	17	9.0	4.9, 22.0	$p < 0.05$[2]
	Mesothelioma	11	4.0	1.0, 14.8	$p = 0.07$[2]
	Small-cell carcinoma	9	4.7	0.0, 8.0	$p < 0.01$[2]
	Others	28	6.2	4.0, 14.0	$p < 0.01$[2]

[1]Statistical significance to primary lung tumors (Kruskal Wallis test); [2]Statistical significance to metastatic tumor group (Wilcoxon test)

Table 3. Spearman Rank Correlation betweeen progelatinase B/proMMP-9 effusion value and C-reactive protein (CRP) effusion and serum values

Analytes Compared	r	n	p
proMMP-9(effusion) v. CRP (effusion)	0.28	191	0.001
proMMP-9(effusion) v. CRP (serum)	0.30	69	0.01
CRP(effusion) v. CRP (serum)	0.83	66	0.001

tive, presumably due to an autoactivation during the renaturation period. In contrast to relatively stable intensities of 72 kDa proMMP-2 bands, the 92 kDa proMMP-9 bands dominate in parainflammatory effusions, are typically less expressed in paraneoplastic effusions, and hardly seen in transudates.

The basic statistics of the main three etiological effusion groups were summarized in Table 1. The non-parametric analysis of variance was performed by Kruskal Wallis test ($p=0.001$) and individual groups were compared by Wilcoxon rank test. The values were statistically significant if paraneoplastic group was compared to parainflammatory group ($p=0.001$) and to transudative group ($p=0.01$), and parainflammatory group to transudate group ($p=0.001$). Standard deviations were 70 (paraneoplastic exudates), 224 (parainflammatory group), and 2.8 ng/ml (transudate group), respectively.

ProMMP-9 concentrations in paraneoplastic effusion subgroups. Despite a significant statistical difference between paraneoplastic and parainflammatory effusions, about 1/5 of the tumor-associated effusions amounted to the median value of parainflammatory pleural fluids. In order to reveal possible tumor infrastructure-linked differences in the MMP-9 expression, tumor-associated effusions were further sorted using histological findings. Concentrations found in metastatic tumors and subgroups of primary tumors were evaluated by a non-parametric test (Kruskal Wallis, $p=0.05$), and individual groups were analyzed by Wilcoxon rank sum test. Median values and lower and upper quartiles were summarized in Table 2. Metastatic tumors were significant ($p<0.05$) to pooled primary tumors and to individual subgroups with the exception of mesothelioma associated effusions. ProMMP-9 levels in metastasis associated subgroup, unlike in other subgroups, did not deviate significantly from inflammation associated group (data not shown). We also used logistic regression analysis attempting to predict the presence of metastatic tumors in carcinoma patients. Using formula $P = 1/(1 + \exp(-1.508 + 0.0015^{*}$ ALL) and a prediction value of 0.5, the test showed 81.3% sensitivity of and 48% specificity. ("P" indicates a metastatic origin).

Correlations between proMMP-9 and CRP in pleural effusions and comparison of pleural and blood serum CRP values. The

Spearman rank correlation test revealed significant correlation between proMMP-9 and CRP values in both effusion and available blood sera. A strong correlation was also found between pleural and blood sera values (Tab. 3).

Discussion

This study shows significant differences in pleural MMP-9 expression among relevant etiological groups of pleural effusions, detecting the highest concentrations in parainflammatory exudates, intermediate in paraneoplastic exudates, and the lowest in transudates. This supports the results of the two previous reports analyzing smaller patient groups [9, 17]. It is also in accordance with our more recent observation that proMMP-9 is upregulated under the conditions of induced pleurisy [18]. Similarly, the analyses of other extracellular fluids, such as synovial fluid [25, 31], amniotic fluid [12, 30], peritoneal fluid [21, 27], and cerebrospinal fluid [2], also demonstrate proMMP-9 elevation under inflammatory conditions.

Despite the statistical significance valid for our paraneoplastic group as a whole, a minor population of paraneoplastic fluids produces considerably higher amounts of the proenzyme than the major group, reaching values typically found in parainflammatory effusions. It is conceivable that in a given population of fluids, an actual distribution of high- and low-MMP-9 expressing fluids may vary. This phenomenon could then clarify conclusions of a report showing the highest MMP-9 expression in paraneoplastic effusions [8].

The proMMP-9 upregulation in metastasis associated pleural fluids is in agreement with the concept that this enzyme is linked to aggresive growth and a metastatic potential of tumors [5, 14, 15, 19, 23, 24, 28]. It appears that a cooperation between different cell types regulating MMP-9 expression is involved in the crucial steps of cancer development. Macrophage-produced MMP-9 promotes angiogenesis, basement-membrane breakdown and tumor-cell egress [26]. The co-cultivation of tumor cells with macrophages leads to enhanced invasiveness of the malignant cells [11]. On the other hand, recent data have expanded the notion that inflammation is a critical component of tumor progression. Inflammatory cells as stromal cells show a variety of pro-tumor actions but under specific circumstances, a massive inflammatory reaction involving leukocyte infiltration leads in some cases even to the elimination of the primary tumor [4].

Inflammatory disorders and other stress conditions are associated with the elevation of acute-phase proteins in blood plasma. Clinical experience shows that the rise in acute-phase proteins, exemplified by C-reactive protein (CRP), is a common finding in cancer patients as well [29]. CRP is produced by hepatocytes and has been detected in pleural fluids. A positive correlation between pleural and plasmatic CRP levels [1, 2, 10, 32], confimed also by this study, points to the plasmatic origin of pleural CRP [33]. We found a positive correlation between pleural proMMP-9 and CRP. This observation suggests that a certain portion of the detected proMMP-9 might be associated with a systemic response to the underlying neoplastic process or to disorders secondary to the primary disease, such as cachexia and/or side effects of a tumor treatment.

To our knowledge, this is the first critical evaluation of proMMP-9 as a biochemical marker in differentiating pleural effusions emphasizing its expression in paraneoplastic effusions. Obviously, its value in the differentiation between paraneoplastic and parainflammatory exudates is limited, due to the heterogeneic expression in the tumor associated group. On the other hand, due to its higher expression in metastases-induced effusions proMMP-9 might be considered a potential indicator of the metastatic origin of lung cancer. The predictive model calculated in this study shows relatively good results. By pointing out the correlation between CRP and proMMP-9 levels, this study also contributes to the ever-growing evidence of the association between cancerogenesis and inflammation.

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