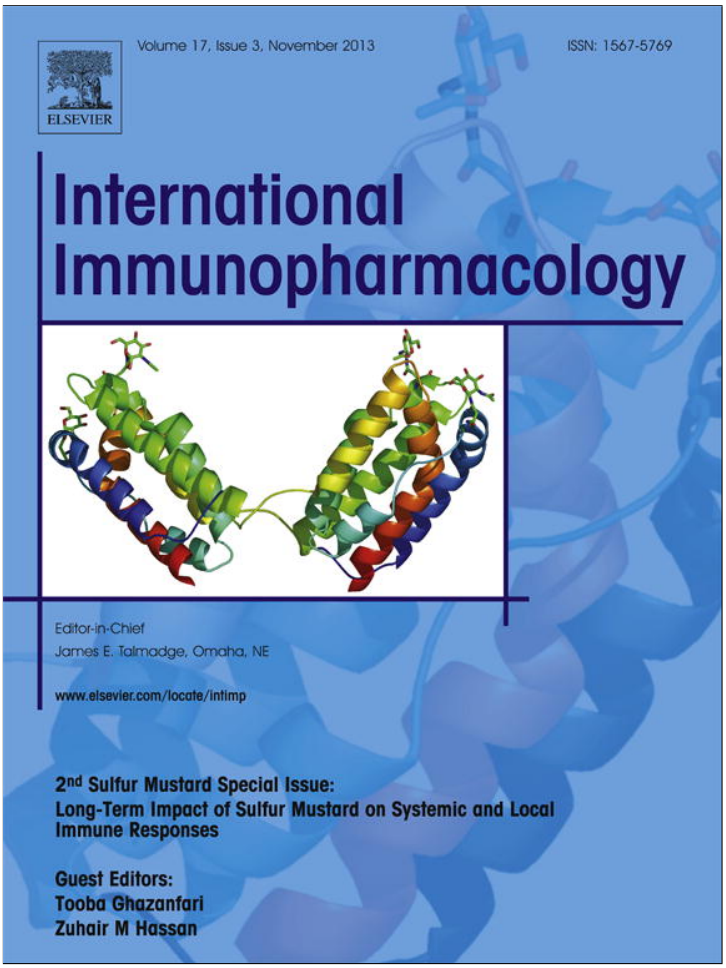

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Serum profiles of matrix metalloproteinases and their tissue inhibitors in long-term pulmonary complication induced by sulfur mustard: Sardasht-Iran Cohort Study (SICS)

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ABSTRACT

Sulfur mustard (SM) is a cytotoxic chemical agent which can cause severe irritation and irreversible damages to body tissues. The effect of SM gas on respiratory tract is one of the main causes of short and long term disabling complications. Matrix metalloproteinases (MMPs) have a critical role in controlling extra cellular matrix remodeling and inflammatory responses in lung tissue and are involved in many various chronic pulmonary diseases. The aim of the present study was to evaluate the possible role of MMPs and their endogenous inhibitors in SM induced lung symptoms in exposed subjects 20 years after exposure. Serum level of MMP-1, MMP-2, MMP-8, MMP-9, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 was measured by ELISA and compared between groups of exposed without any symptoms (control group) and with mild or moderate–severe lung complications. MMP-2 and MMP-9 activity was assayed by gelatin zymography method. There was a significant association between serum level of MMP-1 and severity of lung complications in SM exposed groups. MMP-2 activity was decreased in exposed groups with mild lung complications. TIMPs level was not different in exposed and normal groups. We concluded that increased serum levels of MMP-1 and decreased MMP-2 activity may have roles in pathogenesis and persistence of lung complications in SM exposed victims.

1. Introduction

Previous studies clearly showed that sulfur mustard (SM) exposure induces acute, chronic and late injuries in human organs [1]. The most relevant long-term clinical consequences of SM exposure can be found in eyes, skin, immune systems and respiratory tract [2,3]. Several studies have revealed that respiratory problems are the most common long-term disorder among individuals exposed to SM [4,5]. Furthermore, numerous respiratory dysfunctions have been reported in victims of SM gas, including pulmonary fibrosis, chronic obstructive pulmonary disease (COPD) obliterative bronchiolitis, small airway disease and inflammation [5]. Although there are few reports on respiratory manifestations of long term exposure to SM, little is known about the underlying cellular and molecular mechanisms of chronic SM exposure.

Matrix metalloproteinases (MMPs) are a group of more than 20 zinc-dependent proteolytic enzymes that are involved in the remodeling of the extracellular matrix (ECM) components [6]. In many pulmonary complications, remodeling and destruction of the ECM is a hallmark of the disease [7]. In addition to ECM degradation, MMPs also have important roles in immune system functions. MMPs may regulate responses to exogenous stimulus and contribute to immunopathological processes which lead to abnormal turn-over of the ECM [8]. Expression of MMPs is a complex phenomenon and depends on the biological context and endogenous and/or exogenous regulators. Some MMPs are inducible and the biological milieu like inflammatory conditions changes their expression [9]. In addition to gene expression, the activities of MMPs are also controlled by four natural tissue inhibitors of metalloproteinases (TIMPs) [10]. In a physiological process, there is a precise balance between MMPs and their natural inhibitors. Distortion of this balance can be a cause of many pathological conditions related to ECM remodeling in lungs such as COPD and fibrosis [11].

Considering the important role of MMPs in respiratory system turn-over and remodeling, in the present study we focused on serum concentration and activity of some relevant MMPs (MMP1, MMP2, MMP8, MMP9) and TIMPs in patients with pulmonary complications induced by long-term exposure to SM gas.

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2. Materials and methods

2.1. Study design and participants

The details of study design and methods have been explained previously [12]. We recruited 75 SM-exposed subjects who were male, aged 20–60 years, and from Sardasht. They were categorized into three major subgroups based on the severity of lung problems: 32 SM-exposed but without lung complications, 33 SM-exposed with mild lung complications and 10 patients who were exposed to SM with severe lung complications. This study was approved by the Board of Research Ethics of Janbazan Medical and Engineering Research Center, the Ministry of Health of Iran, and Shahed University. A written informed consent was obtained from all subjects in the study.

2.2. Clinical evaluations

All participants were visited by clinicians. The respiratory signs and symptoms were evaluated by the experienced consultants of the Research Team. Then their respiratory functions were measured by spirometry, according to the American Thoracic Society Criteria. Spirometry was conducted by an experienced nurse using a spirometry device (Chest 801). The classification of the severity of pulmonary complications was carried out according to the Global Initiative for chronic Obstructive Lung Disease (GOLD) [13].

2.3. Serum collection

Peripheral blood was drawn into Vacutainer tubes (BD Biosciences). The sera were separated by 20 min centrifugation at 2000 ×*g* (4 °C), aliquot, labeled and kept frozen at −80 °C until laboratory measurements.

2.4. Measuring serum concentration of MMPs and TIMPs

Human MMP-1, MMP-2, MMP-8, MMP-9, TIMP-1, TIMP-2, TIMP-3, TIMP-4 Quantikine ® ELISA kits (R&D systems, Minneapolis, USA) were used to measure serum MMPs and TIMPs level. Microplates were pre-coated with specific primary antibodies for each MMP or TIMP. The specific secondary antibodies were conjugated to horseradish peroxidase. Stop solution was 2 N sulfuric acid.

2.5. Gelatin zymography

Gelatin zymography was carried out according to the method described elsewhere [14]. Briefly, 60 μg of total serum protein from both healthy control and exposed patients serum were mixed with sample buffer and loaded on a 7.5% polyacrylamide gel containing 1% sodium dodecyle sulfate (SDS) and 1 mg/ml gelatin under nonreducing conditions. Samples were not boiled before electrophoresis. Following electrophoresis, gels were washed with 2.5% Triton X-100 for 1 h at room temperature to remove SDS. The gels then incubated at 37 °C for 24 h in substrate buffer containing 25 mM Tris, pH 7.5 and 5 mM CaCl2 for the development of enzyme activity bands. After that, the gels were stained with Coomassie brilliant blue R-250 in 50% methanol and 10% glacial acetic acid for 30 min and destained in 4% methanol and 8% acetic acid. The gelatinolytic activities were detected as transparent bands against the background of Coomassie brilliant blue stained gelatin. Protein (MMPs) standards were run concurrently for determining the molecular weight of MMP bands (Fig. 1). Gels were scanned and quantification of the bands was determined using NIH Image software.

2.6. Statistical analysis

Analyses of all data were performed with the SPSS software version 16.0. Data were presented as mean. Comparison of inflammatory mediators among groups was performed using the One-way analysis of variance (ANOVA) and Tukey's test for quantitative and normal data. The non-parametric Kruskal–Wallis test was used for the comparison of multiple groups (normal–moderate–severe) of unmoral data and non-parametric U–Mann–Whitney test was used to compare the two groups of data. *P*-value of less than 0.05 was considered as statistically significant.

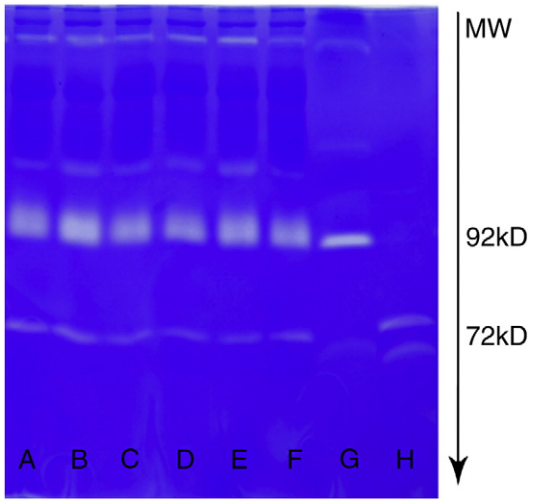

Fig. 1. Panel of representative gelatin zymograms of sera from SM-exposed groups without lung complications (control), with mild and moderate-sever lung complications. Lanes (**A,B**) sera from group without any lung complication; (**D,E**) sera from exposed group with moderate-sever lung injuries; (**C,F**) sera from mild lung complications; (**G,H**) Standards: purified pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa).

3. Results

3.1. Comparison of the serum levels of MMPs in study groups

The data presented in Table 1 shows a significant increase in the serum levels of MMP-1 in the exposed group with moderate to severe pulmonary symptoms compared to that of the control groups (median = 2266 for normal group and, median = 7457 for moderate to severe group, $p = 0.004$). In addition, the difference of MMP level was significant between exposed groups with mild pulmonary symptoms and exposed group with moderate to severe pulmonary symptoms ($p < 0.011$). The difference of serum levels of MMP-2, MMP-8

Table 1
Comparison of serum levels of MMPs between SM exposed group without lung symptoms (Normal) and exposed groups with mild or moderate–severe lung symptoms.

Groups Number	Normal 27	Mild 30	Moderate–Severe 10
MMP-1 (ng/ml)	2266 ± 419.66	2791 ± 308.01	7457 ± 2638.88
p-value1		0.83	0.004
p-value2			0.011
MMP-2 (ng/ml)	100 ± 7.55	103 ± 6.85	104 ± 8.87
p-value1			0.92
MMP-8 (ng/ml)	28 ± 2.31	40 ± 7.04	38.8 ± 6.76
p-value1			0.27
MMP-9 (ng/ml)	1139 ± 260.67	1717 ± 434.94	3355 ± 1458.18
p-value1			0.06

Serum levels of MMP-1, MMP-2, MMP-8 and MMP-9 were assessed in all groups including SM Exposed group without lung symptoms (Normal), SM Exposed group with mild lung symptoms and SM Exposed group with moderate–severe lung symptoms. A comparison was made between each of the exposed groups with the control group. Depicted data are Mean ± SEM.
p-value[1]: comparison of the exposed with mild and moderate–severe lung symptom groups with the normal group.
p-value[2]: comparison of exposed with mild lung symptoms with exposed with moderate–severe lung symptom groups. ng/ml: nanogram per milliliter.

Table 2
Comparison of MMP-2 activity between SM exposed group without lung symptoms (Normal) and exposed groups with mild or moderate–severe lung symptoms.

Groups	Normal	Mild	Moderate–Severe
Number	27	30	10
MMP-2 activity	507.9 ± 39.67	376.1 ± 33.69	324.9 ± 50.91
p-value1		0.03	0.088
p-value2			0.082

Serum MMP-2 activity calculated for all groups and comparison was done between mild and moderate–severe groups with the control group. Data are presented as mean ± SEM.
p-value[1]: comparison of the exposed with mild and moderate–severe lung symptom groups with the normal group.
p-value[2]: comparison of exposed with mild lung symptoms with exposed with moderate–severe lung symptom groups.

and MMP-9 between study groups was not significant. The mean of MMP-9 levels between groups was different but not statistically significant. We have seen wide error bars in MMP-9 levels in the group with moderate to severe pulmonary complications. This may be due the low number of subjects in this group and/or different responses to SM exposure by each individual participant.

3.2. Comparison of the serum levels of TIMPs in study groups

There was no significant association between serum levels of TIMP-1, TIMP-2, TIMP-3 and TIMP-4 between control group and exposed groups with mild or moderate/severe pulmonary symptoms.

3.3. Comparison of MMP-2 and MMP-9 activity in study groups

A significant decrease of MMP-2 activity was observed in exposed groups with mild pulmonary symptoms. Differences in MMP-2 activity were not significant between control group and moderate–severe group and also between mild and moderate–severe subjects. There was no significant association between MMP-9 activities in study groups (Table 2).

4. Discussion

In the present study, we demonstrated that even after a long post exposure period in SM exposed victims, an increased serum level of MMP-1 and decreased MMP-2 activity were associated with the severity of pulmonary complications. To the best of our knowledge, this is the first report considering MMPs level and activity as a biomarker in SM exposed patients with significant pulmonary disorders. However, our results did not show significant differences for the serum levels of MMP-2, MMP-8, MMP-9, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 in SM exposed patients with mild or severe lung complications compared to normal control group.

MMPs are a family of zinc dependent proteases which are involved in extracellular matrix (ECM) degradation and tissue remodeling. Normal lung function requires alveolar support by ECM. Abnormal remodeling of lung ECM leads to loss of function and is a hallmark of lung damage. Because of crucial roles of MMPs in ECM remodeling, fibroblast proliferation and also regulation of immune responses to stimuli, has become an important subject to study [15]. Previous studies have shown that different types of MMPs are involved in chronic pulmonary diseases like COPD and lung fibrosis [16,17]. In our study, MMP-1 level was directly associated with the severity of pulmonary complications in SM exposed patients. MMP-1 is a matrix metalloproteinase that degrades Fibril-forming collagen. In normal conditions, MMP-1 expression is very low but is over-expressed in reactive alveolar epithelial cells [18]. Based on this fact, we can conclude that elevated serum level of MMP-1 is likely due to pathologic changes affecting alveolar microenvironment in SM exposed victims. MMP-1 degrades type1 collagen fibrils and converts it to gelatin which, in turn, can be degraded by other MMPs like MMP-9 and MMP-7 [19]. Others have shown that the concentration of MMP-1 is elevated in plasma, serum, BAL fluid, and lung tissue of patients with idiopathic pulmonary fibrosis (IPF) [20]. In another study, Imai et al. have shown that MMP-1 is produced from alveolar type II cells and over-expressed in patients with emphysema. They suggest that MMP-1 may be an important enzyme involved in the destruction of the lung in the chronic lung diseases [21]. They hypothesized that modification in lung alveolar epithelial cells could induce MMP-1 expression in the parenchyma of the lungs and even after ceasing smoking altered alveolar epithelial cells constitutively express the enzyme and chronic tissue damage may continue [21]. Recent Studies show that SM induces Interleukin 17, which is followed by increase of MMP-1. [22,23]. This phenomenon could be true in SM exposed patients since 20 years after SM expose MMP-1 level is elevated in their serum and is significantly associated with pulmonary complications. A recent autopsy study on SM exposed patients in Iran showed that chronic bronchitis (81%) and progressive pulmonary fibrosis (9%) were the common lung complications in these patients [24]. These results also confirm that chronic lung damage and pulmonary complications are the main cause of morbidity and mortality in long time SM exposed patients. This would further highlight the important roles of MMPs, especially MMP-1 in SM pathogenesis.

In addition to MMP-1, we found that MMP-2 activity is reduced in both mild and moderate–severe pulmonary complication groups but its difference was statistically significant just in mild lung complication group. The lack of association between reduced MMP-2 activity in moderate–severe lung complications group and normal control group may be due to small number of patients that also caused high standard deviations in moderate–severe group. To our knowledge, this is the first study to show significant reduction in extracellular MMP-2 activity in SM exposed patients. Fibroblasts constitutively secrete MMP-2 and indeed in physiological conditions these cells play an important role in ECM remodeling by producing both ECM molecules and ECM-degrading factors. Lower MMP-2 activity levels may cause excessive ECM deposition and disturb gas exchange in alveolar space. La Rocca et al. have shown that exposure to cigarette smoke inhibits extracellular MMP-2 activity in human lung fibroblasts [25]. They concluded that decreased fibroblasts MMP-2 activity may participate in the alteration of proteolysis/antiproteolysis balance and lead to inflammatory chronic lung diseases such as COPD. In the other hand, MMP-2 has a role in deactivating some chemokines and its reduced activity may cause persistent inflammation and subsequently damage to alveolar extracellular matrix. Our results showed reduced MMP-2 activity despite normal serum levels. The ELISA kit used in this study measures total MMP-2 (both pro and active form of MMP-2) and decreased MMP-2 activity may be due to decreased active form of MMP-2. However, more studies are warranted to clarify this notion.

5. Conclusions

Our findings support the use of serum levels of MMP-1 and MMP-2 activities as biomarkers for the severity of lung damage in pulmonary complications in SM exposed patients. This can be used for diagnosis, early detection, and monitoring of disease progression. Moreover, it is conceivable that inhibition of MMP-1 enzyme may be a therapeutic option for the treatment of pulmonary complications induced by SM exposure.

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