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RESEARCH ARTICLE

Bronchoalveolar lavage fluid proteomic patterns of sulfur mustard-exposed patients

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Sulfur mustard is an alkylating agent that reacts with ocular, respiratory, cutaneous, and bone marrow tissues. Main late respiratory complications are chronic obstructive pulmonary disease, bronchiectasis, asthma, and bronchiolitis obliterans. The aim of the present study was to identify differentially expressed proteins in bronchoalveolar lavage (BAL) fluid of control healthy and sulfur mustard-exposed lung disease patients. The BAL protein profile of ten healthy and 30 exposed patients with mild, moderate, and severe conditions (ten males in each group) were separated with 2-D SDS-PAGE and differentially expressed protein spots were successfully identified with MALDI TOF TOF MS. Among the differentially expressed proteins we observed a significant increase in vitamin D binding protein isoforms, haptoglobin isoforms, and fibrinogen especially in exposed moderate and severe lung diseases patients ($p < 0.01$). Moreover, compared with healthy controls, significant decreases was noted in calcyphosine, surfactant protein A, and transthyretin in these patients ($p < 0.01$). Apolipoprotein A1 was detected in all patients' BAL fluid but none of the healthy controls. Furthermore, S100 calcium-binding protein A8 was only detected in BAL fluid of moderate and severe groups. These findings will be useful to improve current methods of monitoring and helps to identify new therapeutic targets for treatment of this complicated illness.

Keywords:
Apolipoprotein A1 / Bronchiolitis obliterans / Surfactant protein A / S100 calcium binding protein A8

1 Introduction

Mustard gas or sulfur mustard (SM), *bis* (2-chloroethyl) sulfide, is a highly reactive agent that alkylates cellular components. SM is a vesicant agent and causes cutaneous blisters, respiratory tract damage, eye lesions, and bone marrow depression. The clinical picture of poisoning is well known from the thousands of victims during World War I and the Iran–Iraq war. In the latter conflict, SM was heavily used and until now about 30 000 victims still suffer from late effects of the agent, such as chronic obstructive lung disease, lung fibrosis, recurrent corneal ulcer disease, and chronic conjunctivitis [1].

The acute toxic effects of mustard gas result from irreversible alkylation of proteins [2] and nucleic acids [3]. Collectively, this causes loss of structural and functional integrity of cells and tissues, resulting in intense pain and burning, with blister formation [4]. Tracheobronchitis usually results several hours after exposure. Other respiratory manifestations can range from bronchospasm and bronchial obstruction to hemorrhagic pulmonary edema. Lethal exposures result in death from respiratory failure,

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Abbreviations: Apo A1, apolipoprotein A1; **BAL,** bronchoalveolar lavage; **BO,** bronchiolitis obliterans; **DLCO,** diffusion capacity of lung for carbon monoxide transfer; **FEV1,** forced expiratory volume in 1 s; **FVC,** forced vital capacity; **HRCT,** high resolution computed tomography; **SM,** sulfur mustard; **SPA,** surfactant protein A; **TCA,** trichloroacetic acid; **Vit D BP,** vitamin D binding protein

secondary pneumonia, and occasional hemorrhagic pulmonary edema [5].

Late complications of mustard gas exposure and main clinical findings include evidence of chronic bronchitis, bronchiectasis, and bronchiolitis obliterans (BO) [6–8]. However, clinical manifestation in lung disorders due to SM is different from other lung diseases. For example, mustard lung is not responsive to corticosteroids, and the course of the disease is not progressive. However, there is no common consensus about the pathophysiological basis of chronic pulmonary disease caused by this chemical warfare agent.

Characterization of proteins secreted into the bronchoalveolar lavage (BAL) fluid provides an opportunity for discovery of diagnostic marker candidates for accurate therapeutic decision-making. Application of BAL to the study of human lung diseases began in the mid-1970s and contributed greatly to knowledge of local immunoinflammatory responses in normal and pathological conditions. Since then, many studies have been dedicated specifically to evaluating BAL cell profile, lymphocyte phenotype, and cell functions in different diseases, with the aim of characterizing the pathological mechanisms involved and providing insights for diagnosis and prognosis [9]. Recent improvements in protein separation methods based on 2-D gel electrophoresis, enlargement of protein databases, and improvements in the identification of proteins using MALDI TOF MS have made the large-scale determination of protein expression practical [10]. There is wide-ranging interest in using the proteomics approach to define markers of lung disease. For instance, recent studies have employed 2-D electrophoresis to study BAL fluid from healthy subjects [11], smokers [12], and patients with a range of pulmonary disorders including cystic fibrosis [13], sarcoidosis [14], and pulmonary fibrosis [15].

Although the respiratory tract lesions represent the major disability after SM exposure, only a few studies have investigated the long-term pathophysiology of SM-induced respiratory diseases, in particular the proteomics. In the present study, we used 2-D electrophoresis followed by MALDI TOF MS, with the aim of identifying differentially expressed proteins involved in exposed patients and healthy controls, which may help in further understanding the nature of long-term effects of mustard gas.

2 Materials and methods

2.1 Patients

According to the American thoracic society classification (http://www.thoracic.org/sections/copd/for-health-professionals/definition-diagnosis-and-staging/spirometric-classification.html) [16] and based on our spirometric and high-resolution computed tomography (HRCT) findings the patients were divided into three groups of mild, moderate, and severe conditions. Each group included ten male patients. A group of ten healthy, male, age-matched individuals was also used as the control. This study was approved by the ethics committee of the research center of Baqiyatallah University of Medical Sciences, and informed consent was obtained from all patients. All participants were free to leave the study at will. The participant patients were suffering from pulmonary disorders due to previous exposure to a single high dose of SM gas during the Iran–Iraq conflict in 1987. Subjects for this study were recruited from surviving residents of Sardasht (a city in western Iran) who sustained exposure to SM during the 1987 attack. Inclusion criteria were as follows: documented exposure to SM and documented diagnosis of chronic pulmonary disease due to mustard gas. Exclusion criteria for the patients and the control subjects were history of a chronic disease (tuberculosis, diabetes, hypertension, heart disease, *etc.*), resection of one or more lobes of the lungs, pneumonia and/or acute bronchitis, smoking cigarettes, or being a substance abuser. None of the patients or control subjects had a history of allergy or asthma. All patients and controls were in a stable condition and none of the participants had been administrated corticosteroids during the 3-month period immediately preceding the studies.

2.2 HRCT and spirometry

Chest HRCT scanning was performed by High Speed Advantage Scanner (General Electric Medical System, Milwaukee, WI, USA). It consisted of five 1.0-mm collimation images obtained during deep inspiration and full expiration, while the patients were in supine position. All chest HRCT scans were reviewed by a radiologist familiar with BO cases. The expiratory images were assessed for the presence of air trapping and its lobar distribution, defined as alteration of normal anterior–posterior lobar attenuation gradients and/or lack of homogenous increase in pulmonary attenuation, resulting in persistent areas of decreased attenuation.

All participants underwent spirometry (by a HI-801 Chest M.I. Spirometer, Tokyo, Japan) before bronchoscopy. The Spirometer was calibrated using the device provided by the manufacturer company. To assess the pulmonary functions, we measured forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC), FEV1/FVC ratio, and diffusing capacity of lung for carbon monoxide transfer (DLCO).

2.3 BAL sampling

BAL was performed with the informed consent of the subjects based on standard procedure [17]. To summarize, the distal tip of the bronchoscope was wedged into the middle lobe or lingular bronchus. A total of 150 mL of warm (37°C), sterile, pyrogen-free saline solution was instilled in 30 mL aliquots and serially recovered by gentle aspiration.

The first collected aliquot was used for the microscopic and cultural examination of common bacteria and fungi, and of direct acid-fast bacilli smears (Kinyoun method) and cultures. All the other aliquots were pooled and used to assess the total and differential cell counts (performed on cyto-centrifuged preparations with the use of May–Grünwald–Giemsa and Papanicolaou staining). Samples were immediately filtered through gauze, and then centrifuged at $800 \times g$ for 10 min, supernatant was removed, and one tablet of complete protease inhibitor cocktail (Roche, Mannheim, Germany) was added and were promptly frozen in aliquots and stored at $-80^{\circ}C$ until use.

2.4 Trichloroacetic acid protein precipitation

Total protein in BAL fluid was determined by the bicinchoninic acid assay and employed bovine albumin as standard (Pierce, Rockford, IL, USA). BAL fluid protein was desalted and concentrated using trichloroacetic acid (TCA) procedure according to Jiang *et al.* [18], with slight modification. Briefly 60% TCA was added to the BAL fluid bringing to the final optimized concentration of 12% w/v TCA. The mixture was vortexed and incubated overnight on ice and centrifuged at $12\,000 \times g$, $4^{\circ}C$ for 15 min. The supernatant was removed and pellet was dispersed in ice-cold acetone. Samples were vortexed and incubated at $-20^{\circ}C$ for 30 min and then centrifuged as above. The acetone-containing supernatant was removed and the pellet was air-dried. For 2-D gel electrophoresis, the pellet was suspended in sample buffer, consisting of 7 M urea, 1 M thiourea, 20 mM Tris, pH 7.5, 4% w/v CHAPS, and centrifuged at $10\,000 \times g$ at room temperature for 5 min. Supernatants were taken for 2-D gel analysis. Protein concentration in the recovered samples was determined using a modification of the method described by Bradford [19].

2.5 Polyacrylamide 2-D gel electrophoresis

TCA-precipitated protein samples (500 μg) were reconstituted in rehydration buffer [7 M urea, 1 M thiourea, 2% wt/vol CHAPS, 0.5%, pH 3–10, IPG buffer matching the IPG strip, 45 mM DTT, and a trace of bromophenol blue] to a final volume of 450 μL. The samples were incubated at room temperature for 30 min, and then used to rehydrate 24-cm IPG strips (Bio-Rad) with a pH 3–10 non-linear or pH 4–7 linear gradient. The strips were passively rehydrated overnight at room temperature in a re-swelling tray. First-dimension IEF was carried out at $20^{\circ}C$ in a PROTEAN IEF Cell (Bio-Rad) by the following protocol: 250 V for 15 min, 8000 V for 3 h, then at 10 000 V for a total of 60 000 Vh, limited by a maximum current of 50 μA *per* gel. Then, the focused IPG strips were incubated for 15 min in equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS) complemented with 1% w/v DTT, followed by incubation for 15 min in equilibration buffer supplemented with 2.5% w/v iodoacetamide to prevent thiol reoxidation. Next, the proteins were separated in the second dimension on homogeneous 12 or 15% polyacrylamide gels in an Ettan DALTsix electrophoresis unit (GE Healthcare, Uppsala, Sweden). The IPG strips were placed on the top of polyacrylamide gels ($1 \times 200 \times 260$ mm) and sealed with a solution of 1% w/v agarose containing a trace of bromophenol blue. Gels were run in a running buffer, containing 25 mM Tris, 192 mM glycine, and 0.1% w/v SDS at 2 W/gel for 45 min, followed by 12 W/gel at $20^{\circ}C$ until bromophenol blue had migrated to the bottom of the gel. Gels were fixed overnight in acetic acid, methanol, water, 10:45:45 v/v and stained with colloidal CBB G-250 [20].

2.6 Data analysis of 2-D gel profiles of BAL fluid proteomics

For image analysis, the CBB-stained gels were scanned in transmission scan mode using Bio-Rad GS-800 calibrated densitometer at a resolution of 600 dots *per* square inch. The scanned gels were saved as TIF images for subsequent analysis. Gel images were analyzed using ImageMaster 2D Platinum software (version 6.0; GE Healthcare). Protein spots were detected automatically. Manual spot editing or deleting (of artifacts) was performed when necessary. To measure the density of protein spots on CBB-stained gels the volume of each spot was divided by the total volume of all spots of the same gel. Since this method of normalization produces extremely small values, the result was multiplied by scaling factor of 100, which produce spot percentage volume. For those proteins with multiple spots in the gel, such as surfactant protein A (SPA), the mean pixel volumes of sum of the individual spots from isoforms were used for analysis. Only those spots that were reproducibly and significantly ($p < 0.05$) different in intensity or not matched in the patients compared with controls were used for analysis. These spots were carefully inspected for inappropriate matching, staining artifact, or bad spot detection and conserved for analysis.

2.7 Analysis of tryptic digests with MALDI TOF TOF MS

Protein spots of interest were aseptically picked under laminar flow hood. Gel plugs were washed twice with double distilled water, followed by 50% ACN in 50 mM ammonium bicarbonate and pure ACN. The gel plugs were dried in speedVac evaporator and were rehydrated for digestion with 0.1 μg of sequencing-grade modified trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate and incubated overnight at room temperature. The resulting peptides were concentrated on a Zip Tip micro-purification column and then 1 μL of this solution was directly spotted onto the MALDI target. Then, 1 μL of a saturated CHCA solution in 70% CH_3CN was added and

mixed with the sample by aspirating the mixture five times. The samples were allowed to dry on the target for 10–15 min before measurement in MALDI TOF. MALDI TOF MS was performed using Bruker Autoflex III MALDI TOF/TOF instrument (Alphalyse, Denmark). An external peptide calibration standard containing Angiotensin II ($[M+H]^+$ 1046.54), Angiotensin I ($[M+H]^+$ 1296.68), Substance P ($[M+H]^+$ 1347.74), Bombesin ($[M+H]^+$ 1619.82), ACTH clip 1–17 ($[M+H]^+$ 2093.09), ACTH clip 18–39 ($[M+H]^+$ 2465.20), and Somatostatin 28 ($[M+H]^+$ 3147.47) (Bruker Daltonics) was used to calibrate the instrument. Spectra were acquired in positive reflector mode. A list of peptide was obtained using algorithm SNAP (Flexanalysis 3.0, build 54, Bruker Daltonics). Any number of peaks that the SNAP algorithm finds as a mono-isotopic peak were used for the database search. Intensity and area was not considered. The most intense peaks were selected for MS/MS scan to obtain as many CID spectrums as possible. The MS and MS/MS spectra were combined and used for a database search using the MASCOT software (Matrix science, version 2.2.03) for database searches with the selection of following criteria: Database search program: NRDB1 (6655203 protein sequence), species of origin *Homo sapiens*, peptide ion mass tolerance 60 ppm, MS/MS tolerance 0.2 Da, peptide cut-off of 25, and digestion by trypsin allowing for no more than one missed cleavage. The accuracy of mass detection was MH^+ of 0.01 assuming possibility of modification of cysteine by acrylamide and oxidation of methionine. Proteins identification was based on a combination of the PMF with peptide masses and several MS/MS spectra of selected peptides in each MALDI MS spectrum. The protein and protein isoforms shown on the identification list are based purely on the human protein database accession that gives the highest MASCOT score. To the extent that the different sequence isoforms are represented in individual database accession numbers, these have been considered in the database search. Other modifications and isoforms are not considered in this analysis. The positive protein identification was based on a probability-scoring algorithm (www.matrixscience.com) and the 95% confidence level for positive identification was a score of 80.

2.8 Statistical methods

All data are representative of at least three independent experiments with ten individuals in each group and are expressed as means ± SEM. Statistical testing used analysis of variances followed by a *post hoc* Dunnett's test, tow-tailed. The criterion for statistical significance was $p < 0.05$ for all comparisons.

3 Results

3.1 Patients' data

BAL was performed in 40 participating male subjects, including 10 healthy controls and 30 patients suffering from mustard gas lung injuries. No clinical or microbiological evidence of bacterial or fungal infections was ever found in the included patients and controls. The mean (SEM) age and clinical findings of the patients and the controls are presented in Table 1.

3.2 Chest HRCT and spirometry findings

Chest HRCT findings of bronchiectasis, air trapping, mosaic parenchymal attenuation, and bronchial wall thickening were seen in our patients. Air trapping was defined as the presence of a radiolucent region of the lungs on expiratory images. The degree of air trapping was assessed by

Table 1. Clinical characteristics, spirometric, and hematologic findings of exposed patients and control subjects

Variables	Healthy	Mild	Moderate	Severe
Age (year)	36.8 ± 3.9	40.3 ± 2.6	43.0 ± 1.8	42 ± 2.8
Lung function tests:				
TLC (%)		83 ± 5.6	91 ± 5.2	76 ± 5.1
RV (%)		130 ± 16	146 ± 15	124 ± 19
FEV1 (%)		84 ± 7.1	65 ± 11	41 ± 5.6**
FVC (%)		84 ± 5.1	68 ± 10.5	57 ± 3.6*
FEV1/FVC		100 ± 7.9	94 ± 9.5	70 ± 5.7*
DLCO (%)		108 ± 6.1	101 ± 12	77 ± 8.4*
PaO_2 (mm Hg)		72 ± 3.1	62 ± 7.9	70 ± 2.9
Pa CO_2 (mm Hg)		36 ± 1.2	37 ± 1.5	38 ± 1.3
BAL cell counts:				
Macrophages (%)	85.4 ± 2.1	70.5 ± 10.8	55.6 ± 13.8	39.9 ± 15.5*
Lymphocytes (%)	4.5 ± 0.5	6.5 ± 2.7	5.8 ± 1.0	3.7 ± 1.2
Neutrophils (%)	10.6 ± 1.7	22.1 ± 11.0	28.3 ± 13.0	57.1 ± 15.5**
Eosinophils (%)	0.1 ± 0.03	0.1 ± 0.04	0.1 ± 0.03	0.1 ± 0.05
Protein in BAL (μg/mL)	75.6 ± 5.1	80.1 ± 10.8	90.2 ± 6.1	104.2 ± 16.8

Data are mean ± SEM. Difference between the groups were calculated by Dunnett's *post hoc* test and marked by $*p < 0.05$, $**p < 0.01$, TLC, total lung capacity; RV, residual volume.

comparing end-inspiratory and expiratory images at similar anatomic levels and at each of the three levels. These findings were compatible with our previous studies, demonstrating BO in all the cases [8, 21]. Staging of the patients to mild, moderate, and severe groups was based on this and spirometric data. The mean difference in FEV1, FVC, FEV1/FVC, and DLCO was found to be significant between the study groups (p value is reported in Table 1). Although the mean difference in PaO2 (mm Hg) seems to have a difference in the moderate and severe group, but it was not significantly different ($p > 0.05$).

3.3 BAL fluid protein profiles

To investigate the protein expression pattern in the BAL fluids of exposed and control subjects, 2-D PAGE was run. Initially, for first dimension, 24 cm IPG strips with pH values in the range of 4–7 were used to obtain a greater resolution in protein separation and also eliminate the interference of basic Ig (IgG heavy and light chains). The proteins were resolved in a homogeneous 12% acrylamide gel in the second dimension (Fig. 1). A typical 2-D gel representing the BAL fluid of healthy subjects and patients had about 300 spots detected by colloidal CBB staining. Figure 1 shows the protein pattern of BAL fluid of a healthy (Fig. 1A), mild (B), moderate (C), and severe (D) lung damage of SM-exposed patients. Images from other healthy volunteers and patients were similar (data not shown). We analyzed the differences in the BAL protein pattern, comparing the gels of the group of diseased and healthy controls. The analysis of protein pattern of the BAL fluid was focused on those protein spots, which showed differences, comparing the patient and the controls, and which could be identified with sufficient reliability. They were compared with ImageMaster 2D software and indicated only

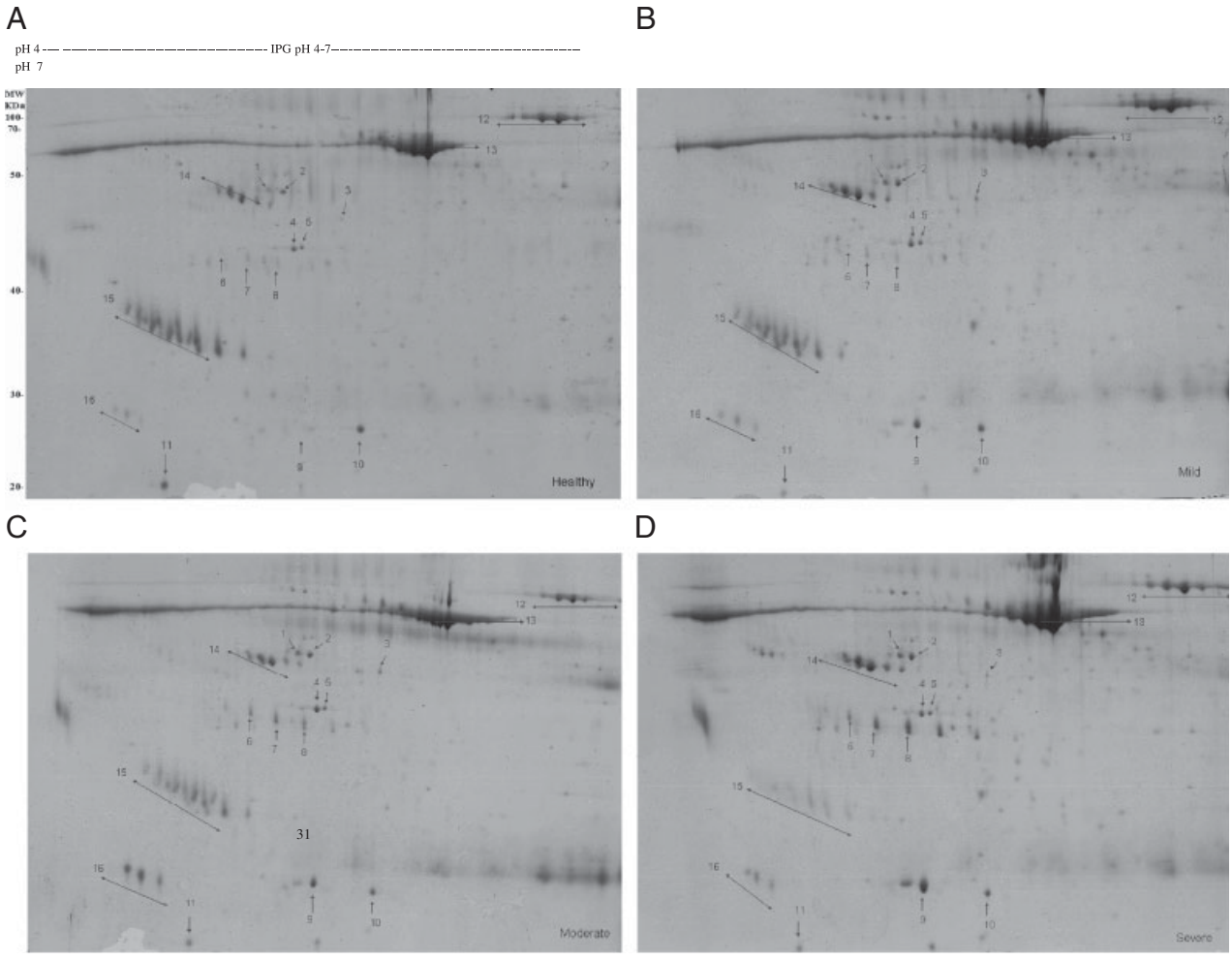

Figure 1. Representative 2-D protein patterns of BAL fluid from healthy and lung patients exposed to SM. Each gel represents as normal (A) mild (B), moderate (C), and severe (D) condition. Sample preparation and 2-D analysis were performed as described in Section 2. Proteins (500 μg) were separated using linear IPG strips, pH 4–7, followed by 12% SDS-PAGE and were detected by colloidal CBB staining. Spots number (1–11 and spot 15) indicate the differentially expressed proteins as listed in Table 3.

protein results in all cases (100%) with the same condition. In the second phase we used 24 cm, 3–10 non-linear pH gradient IPG strips to cover broad pH range and 15% SDS-PAGE in second dimension to resolve low-molecular-weight proteins (Fig. 2). As shown in Fig. 1, the last spot (spot 11) is calcyphosine at the bottom of the gel; also in Fig. 2 spot 17 is related to calcyphosine. Compared with 12% gel in Fig. 1, most of the lower molecular weight proteins are shown in 15% acrylamide gel in Fig. 2 (spots 18–20). In order to identify the altered proteins in the patients and controls all the differentially expressed protein spots and other proteins spot such as actin isoforms, lysozyme, and GST, which were not significant, were subjected to MALDI TOF TOF MS analysis. All selected proteins and their isoforms were subsequently identified by PMF and MS/MS analysis. MASCOT score value and percent coverage for all analyzed proteins were higher than albumin, which was used as quality control internal standards. Table 2 lists the identities

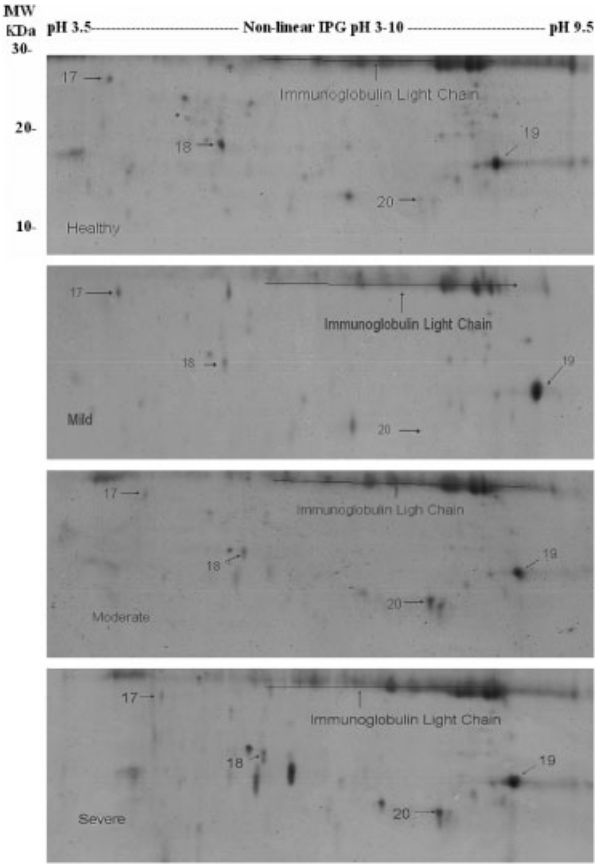

Figure 2. Difference in 2-D gel protein patterns of control and patients. Gels from top to bottom are: from a normal (healthy), SM-exposed patient (mild) SM-exposed patient (moderate), and exposed patient (severe) performed using non-linear IPG strips, pH 3–10, followed by 15% SDS-PAGE. Proteins were detected by colloidal CBB staining. Proteins showing statistically significant differences in percentage volume in the three diseases are indicated with spots number (17, 18, and 20) and are presented in Table 3.

of the proteins and their isoforms, which were analyzed in this experiment using MALDI TOF MS. Figures 1 and 2 show the location of these protein spots in the 2-D gels of exposed patients and controls. In addition, the position of all spots especially spot 12 (transferrin isoforms), spot 13 (albumin isoforms), spot 14 (α1 antitrypsin isoforms), spot 15 (SPA isoforms), and spot 16 (Ig joining chain isoforms), which were not analyzed by MADI TOF MS (in this experiment), was confirmed and verified against the spot positions found in published BAL fluid 2-D maps [22].

3.4 Over- and under-expressed proteins in BAL fluid

Several proteins or protein isoforms (Table 2) were found in significantly different amounts (expressed as % volume) in BAL fluids of healthy controls and patients groups. All differentially expressed proteins and their isoforms are presented in Table 3 except actin isoforms that were not significant among the different experimental group and are presented for relative volumes of spots comparison. Data shows statistical analysis of the percentage volume of the diseased and healthy controls, with p values. Vitamin D binding protein (Vit D BP) isoforms (spots 1 and 2) were significantly increased in all diseased groups and this increase was prominent in moderate and severe *versus* mild group ($p < 0.05$). The 3-D view of the over-expression of Vit D BPs spots (%volume) in all groups are compared with constitutively expressed actin isoforms spots as shown in Fig. 3. The other differentially expressed protein values are also compared with actin isoforms. The results shows that actin isoforms are expressed equally in all groups, but expression of Vit D BP isoforms are significantly different (Fig. 3).

Fibrinogen (spot 3) had a pale not detectable spot in healthy controls but was expressed in all patients. Haptoglobin isoforms (spots 6–8) were significantly over-expressed in moderate and severe patients in contrast to mild and healthy controls ($p < 0.01$). Calcyphosine (spots 11 in Fig. 1 and spot 17 in Fig. 2) was significantly under-expressed in all patients compared with healthy controls. Furthermore, transthyretin (spot 18) was reduced in BAL fluid of patients, particularly in moderate and severe groups ($p < 0.01$). For other proteins, multiple isoforms were evident. For instance, in BAL fluid of all healthy controls and patients there were up to 12 distinct spots for SPA (pI 4.1–5.0; 30–37 KDa; spot 15). Significant decreases were noted for these protein isoforms among different groups of patients, especially in the severe group (Fig. 1D).

In our experimental conditions, comparison of the gels indicated certain proteins presents in one group and not in the others. S100 calcium binding protein A8 (calgranulin A) was only detected in the moderate and severe patients but not in mild patients and healthy controls (Figs. 2C and D). Moreover, Apolipoprotein A1 (Apo A1) could not be detected

Table 2. Comparison of peak numbers, sequence coverage (in %), and MASCOT scores of BAL fluid proteins spots

Spot no.[a)]	Protein name	Database ID[b)]	MW (kDa) /p*I*	MS/MS[c)]	MALDI/MS[d)]	Score[e)] value	Coverage[f)] (%)
1	Vit D BP	gi32483410	52.9/5.32	3/5 [73, 38, 58]	6/12	311	24
2	Vit D BP	gi32483410	52.9/5.33	4/6 [76, 69, 61, 53]	7/15	427	32
3	Fibrinogen	gi182439	49.5/5.61	4/5 [90, 38, 92, 44]	6/12	343	28
4	Actin	gi113370	41.6/5.31	3/4 [147, 48, 127]	9/17	478	41
5	Actin γ1	gi4501887	41.8/5.41	3/4 [58, 117, 53]	8/15	383	40
6	Haptoglobin	gi3337390	38.2/6.13	4/4 [35, 56, 87, 35]	6/14	296	34
7	Haptoglobin	gi3337390	37.9/6.14	4/4 [63, 51, 53, 42]	6/11	216	34
8	Haptoglobin	gi3337390	37.3/6.15	4/4 [56, 53, 57, 65]	7/12	246	34
9	Apo A1	gi4557321	30.8/5.56	3/4 [50, 80, 39]	7/16	297	39
10	GST	gi4504183	23.3/5.43	3/4 [79, 134, 80]	4/9	393	45
11	Calcyphosine	gi4757908	20.9/4.74	4/5 [43, 44, 73, 67]	6/13	345	53
17	Calcyphosine	gi4757908	20.9/4.74	3/3 [45, 77, 34]	5/11	179	47
18	Transthyretin	gi114319005	14.9/5.52	2/2 [42, 74]	4/14	140	19
19	Lysozyme	gi4557894	16.5/9.38	3/3 [32, 106, 33]	5/8	223	47
20	S100 A8	gi21614544	10.8/6.51	3/3 [87, 44, 90]	6/12	308	47

a) Spot no. related to the annotation in Figs. 1 and 2. Number of peaks is given as matched to the protein/total number of SNAP algorithm selected peaks in the spectrum. Mass quality was assessed using transferrin and BSA as quality control internal standards. Score/coverage for transferrin and BSA was 603/43 and 126/15, respectively.
b) NRDB1 (6655203 protein sequence).
c) The column refers to the results of the MALDI-MS/MS analysis to the number and Mowse scores (in brackets) of assigned peptides.
d) The column refers to the results of the MALDI-MS PMF analysis of the number of assigned peptides to total number of measured peptide masses picked with SNAP algorithm.
e) MASCOT score value indicates the quality of database search results.
f) Sequence coverage refers to the observed sequence coverage of the assigned protein.

Table 3. Up- and down-regulated proteins and in SM exposed patients *versus* healthy control[a)]

Spot no.[b)]	Protein	Healthy	Mild	Moderate	Severe
1	Vit D BP	20 ± 1.4	$27\pm1.8^{*}$	$34\pm4.2^{**}$	$32\pm1.6^{**}\uparrow$
2	Vit D BP	32 ± 4.1	$42\pm3.4^{*}$	$48\pm3.2^{**}$	$51\pm4.7^{**}\uparrow$
3	Fibrinogen	Not detectable	16 ± 1.2	18 ± 2.1	$18\pm1.8\uparrow$
4	Actin	44 ± 3.8	45 ± 2.7	47 ± 3.1	$39\pm3.2\leftrightarrow$
5	Actin γ1 propeptide	21 ± 2.3	20 ± 3.0	19 ± 2.8	$22\pm2.5\leftrightarrow$
6	Haptoglobin	2 ± 1.2	5 ± 3.6	$17\pm3.9^{**}$	$23\pm4.4^{**}\uparrow$
7	Haptoglobin	4 ± 1.1	7 ± 2.3	$23\pm3.8^{**}$	$41\pm3.2^{**}\uparrow$
8	Haptoglobin	5 ± 3.2	6 ± 3.1	$32\pm4.2^{**}$	$48\pm5.9^{**}\uparrow$
9	Apo A1	Not expressed	61 ± 8.2	77 ± 4.1	$83\pm6.2\uparrow$
11	Calcyphosine	57 ± 3.0	$19\pm3.9^{**}$	$16\pm2.7^{**}$	$18\pm2.1^{**}\downarrow$
15	Surfactant protein A	98 ± 12	$65\pm5.8^{*}$	$51\pm7.5^{**}$	$21\pm3.5^{**}\downarrow$
17	Calcyphosine	29 ± 4.0	$19\pm3.8^{*}$	$11\pm4.2^{**}$	$9\pm3.5^{**}\downarrow$
18	Transthyretin	25 ± 3.6	19 ± 4.3	$15\pm3.1^{**}$	$14\pm1.2^{**}\downarrow$
20	S100 Protein A8	Not expressed	Not expressed	37 ± 3.2	$42\pm2.8\uparrow$

a) Data are mean$\pm$SEM of the % volume of Protein spots on the 2-D gels multiplied with 100. Difference between the groups were calculated by Dunnett's *post hoc* test and marked by $*p<0.05$, $**p<0.01$, compared with healthy controls. Significantly increased ($\uparrow$) *versus* control. Significantly decreased ($\downarrow$) *versus* control. No significant differences ($\leftrightarrow$).
b) Spot numbers corresponding to those in Figs. 1 and 2.

in healthy controls but in 100% of patients with mild, moderate, and severe conditions (Figs. 1B–D). Finally, the Ig joining (IgJ) chain is a small polypeptide that regulates IgA and IgM polymer formation and is expressed by mucosal and glandular plasma cells [23]. In healthy and all patients there were three distinct spots visible (Figs. 1A–D spot 16).

4 Discussion

In this study, we present the first differential proteomic analysis of BAL fluid from SM-exposed patients and healthy controls. Some of these changes include the over-expression of Vit D BP, fibrinogen γ chain, haptoglobin isoforms, and under-expression of calcyphosine, transthyretin, and SPA

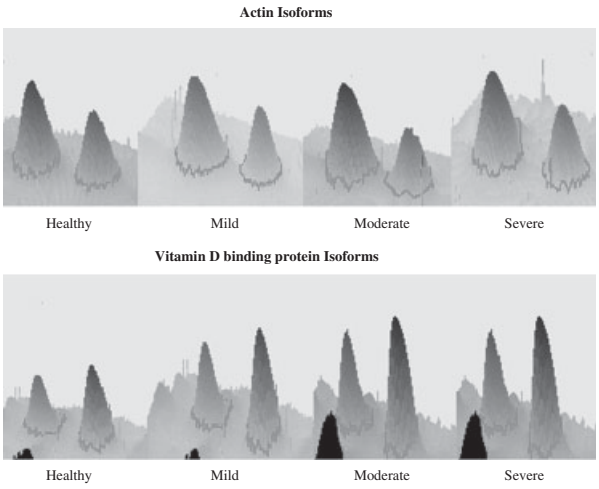

Figure 3. Expression levels of Vit D BP and actin. Protein spots expressed differently from controls and patients BAL fluid as shown on Fig. 1 2-D gels. Upper track are actin isoforms (spots 4 and 5) and lower track are Vit D BP isoforms (spots 1 and 2). The 3-D images of differential spots between controls and patients were analyzed by ImageMaster 2D Platinum software.

isoforms. BAL fluid contains a wide variety of proteins that are either locally released by epithelial and inflammatory cells or through plasma exudation. Because of the diverse origin of BAL proteins, analysis of BAL fluid may reveal important pathologic mediators and may enable more accurate characterization of many lung diseases at the molecular level. The analysis of BAL fluid proteome can potentially provide important information about changes in protein expression and secretion during the course of pulmonary disorders. In the current study we have isolated some proteins that can lead to a novel insight into the pathogenesis of mustard lung.

Vit D BP is a multi-functional plasma protein with many important functions. These include transport of vitamin D metabolites, control of bone development, binding of fatty acids, sequestration of actin, and a range of less-defined roles in modulating immune and inflammatory responses. Also, systemic administration of Vit D BP can inhibit the rate of tumor growth of various solid tumors and, in some cases, can cause regression of established tumors by inducing apoptosis [24]. Apoptosis and the removal of apoptotic cells (termed *efferocytosis*) are associated with the regulation of normal lung structure. Processes that disrupt this balance have the potential to alter normal cell turnover, resulting in the developing of lung pathology and disease [25]. Magi *et al.* [26] have used 2-D electrophoresis to analyze the protein composition of BAL fluid from patients with sarcoidosis and idiopathic pulmonary fibrosis. In sarcoidosis they found an increase in the amount of several plasma proteins, while in idiopathic pulmonary fibrosis they observed a statistically significant increase in low-molecular-weight proteins, many of which are involved in inflammatory processes such as calgranulin but no change in Vit D BP. The β-actin gene is highly conserved in eukaryotes and is expressed in most cell types. Because of constitutive expression of actin mRNA and protein, it is used as an internal standard in gene expression and Western blotting experiments [27]. Hence, in this experiment the expression levels of differentially expressed protein spots were compared with actin isoforms (spots 4 and 5).

Calcium-binding proteins seem to be the main proteins involved in the process of SM pathogenecity. In this study we found two Ca^{2+}-binding proteins, calcyphosine, which was significantly decreased and S100 calcium-binding protein A8, which was significantly increased in moderate and severe groups. The similar patterns of increase in S100 A8 and decrease in calcyphosine mRNA expression have been obtained with the gene expression changes occurring *in vivo* in the repairing healthy human airway epithelium in response to injury [28]. The S100 gene family is composed of at least 20 members that share a common structure defined in part by the Ca^{2+}-binding EF-hand motif similar to calmodulin [29]. The mechanism(s) underlying the SM-induced rise in intracellular Ca^{2+} has not been fully elucidated. Recently Ruff and Dillman [30] have reviewed the Ca^{2+} signaling in SM exposed tissues and cells. Two mechanisms have been proposed for the rise in Ca^{2+} levels in relation to apoptosis. The first mechanism involves protein kinases signaling pathways, which lead to the activation of phospholipase C and the generation of inositol triphosphate, which acts on Ca^{2+} channels to release Ca^{2+} from intracellular stores. The second mechanism involves oxidative stress in which reactive oxygen species generated by toxicant exposure react with Ca^{2+} transport channels in the endoplasmic reticulum, mitochondria, and cell membrane. S100 protein isoforms are implicated in the immune response, differentiation, cytoskeleton dynamics, enzyme activity, Ca^{2+} homeostasis, and growth [31]. Also, evidence has recently accumulated that in addition to other functions revealed that this family of proteins has a broad spectrum of activity in regulating apoptosis and tissue remodeling [32]. It was also shown that S100 A8 and Apo A1 both increased in BAL fluid in endotoxin-challenged and patients with acute respiratory distress syndrome but not in healthy controls [33]. However, the exact physiological role(s) of this protein complex awaits further investigation. In our experimental condition S100 A8 was only detected in moderate and severe patients. Similar results were obtained in previous studies too. Watteiz *et al.* used 2-D electrophoresis for BAL fluid proteomics analysis. Their data showed that S100 A8 (calgranulin A) was not found in healthy normal subjects but it was detected in silver-stained gels of sarcoidosis and idiopathic pulmonary fibrosis patients [15]. de Torre *et al.* [33] used non-linear Immobiline gradient 3–10, followed by 12% SDS-PAGE and protein spots were detected by silver staining. Their results show that S100A8 and Apo A1 were detected in the endotoxin-treated BAL only but not in healthy controls. S100 A8 is mostly produced by neutrophils and induces neutrophils

chemotaxis and adhesion [34]. Our data show that neutrophils significantly increased in sever patients. This might be due to increase in S100 A8 level or *vice versa*. It seems that this protein may have a protective role against lung tissue damage. Furthermore, it could help differentiate the severity of the damage in exposed patients. Calcyphosine, another calcium-binding protein, is involved in cell growth and differentiation and may regulate essential cell functions like proliferation and differentiation as well as cell degranulation [35]. Decreased levels of this protein might cause inhibition of these processes. Similar reduction was obtained in BAL fluid of sarcoidosis patients [14].

Surfactant proteins are synthesized by alveolar type II cells and stored as intracellular inclusion organelles called ''lamellar bodies'' [36]. Particularly, SPA is involved in a range of functions including clearance of bacteria, fungi, and apoptotic and necrotic cells, down-regulation of allergic reaction and resolution of inflammation [37]. In the current study, significant reduction in SPA isoforms was noticed in patients and this was correlated with the severity of pulmonary dysfunction. Other investigators also have reported semi-quantitative relative decreases in the intensity of SPA in patients with hypersensitive pneumonitis and idiopathic pulmonary fibrosis [15]. We assume that not only SM directly damages the lung tissues, but also it may play a destructive role on the lung protective factors.

A significant increase in Apo A1 and haptoglobin isoforms was observed. The increase in these proteins was associated with the severity of pulmonary dysfunction. In our experimental conditions, Apo A1 was not detected in any of the healthy control BAL fluid, but was expressed in all of the patients. It seems that its expression might be induced in lung tissues in which the mild group with low damage also showed increase in Apo A1 expression. In contrast, S100 calcium-binding protein dominantly expressed in moderate and severe groups but not in the mild group. Several studies have suggested an anti-oxidant effect for these two proteins [38, 39], which confirms our latest theory of considering the oxidative–antioxidative imbalance in the pathophysiology of SM-induced pulmonary lesion [40].

Patients with SM-induced pulmonary disorder often receive bronchodilators, corticosteroids, immunosuppressive agents, antibiotics, mucolytics, long-term oxygen therapy, and physiotherapy. But these different treatments are not as effective as predicted and also have known adverse effects. Also, our HRCT findings are compatible with our previous study, demonstrating BO in these patients [8]. BO is considered to be a complex multifactorial process involving immune-mediated and allo-immune-dependent tissue injuries and aberrant tissue repair responses or remodeling [41]. Pulmonary function test, especially the DLCO index (Table 1), proves that these patients do not suffer from pulmonary fibrosis. Also, arterial blood gas samples taken from the patients do not exhibit hypoxia or hypercapnia even in the severe cases of respiratory failure, which rules out the presence of pulmonary emphysema.

In conclusion, this study involved in comprehensive, proteomics analysis of patients exposed to SM gas, which resulted in identification of a number of differentially expressed proteins. To our knowledge, this is the first study of BAL fluid proteomics in SM-exposed subjects. In our experimental condition, the patterns of differentially expressed proteins are somehow different from other lung diseases. It seems that obliterated lung has an aberrant tissue remodeling, which results in abnormal tissue architecture. Increase in S100 A8 and decrease in calcyphosine calcium-binding proteins could be a biomarker for SM-induced lung damage and needs further studies. Furthermore, conclusion from this study could enable us to categorize the patients and predict which patients will develop severe pathologic condition, which may lead to appropriate treatment protocols.

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