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High mobility group box 1 protein in bronchoalveolar lavage fluid and correlation with other inflammatory markers in pulmonary diseases

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Abstract. *Objectives:* Analysis of new markers in bronchoalveolar lavage fluid (BALF) provides new insights into the immunopathogenesis and may be helpful in differential diagnosis of lung diseases. High mobility group box 1 protein (HMGB1) is a non-histone nuclear protein and its release into the extracellular environment may be associated with the inflammatory response. The aim of the study is the analysis of HMGB1 in BALF, correlations with other markers of inflammation and differences in extracellular HMGB1 levels in various lung diagnoses. *Methods:* The concentration of HMGB1 was tested by an Elisa test. We calculated correlations with other inflammatory markers (leukocytes, total protein, albumin, IgG, IgA, IgM, C3 complement component, alpha-2macroglobuline, CD3, CD4, CD8, TREM-1 and TREM-2) and specified HMGB1 level in various diagnoses. *Results:* A positive correlation was found between the level of HMGB1 and total protein levels (p=0.0001), albumin (p=0.0058), IgA (p=0.011), IgM (0.0439) and TREM-2 (p=0.0188). Conversely, a negative correlation was revealed between HMGB1 and TREM-1 (p=0.0009). HMGB1 level varied in different diagnoses: the highest level was detected in QuantiFERON TB-positive subjects (median: 30.2) and hypersensitivity pneumonitis (median: 33.2), followed by pulmonary sarcoidosis (median: 16.8) and idiopathic pulmonary fibrosis (median: 8.8). *Conclusion:* HMGB1 correlates with other inflammatory markers tested in BALF. Its level varies in different lung diagnoses. *(Sarcoidosis Vasc Diffuse Lung Dis 2018; 35: 268-275)*

Key words: sarcoidosis, tuberculosis, DPLD, hypersensitivity pneumonitis

Introduction

HMGB1 (High mobility group box 1) was first identified in 1973 as a non-histone nuclear protein (1). Nuclear HMGB1, as an architectural factor, sus tains chromosome structure and stability, is master of DNA repair and fulfils many other important intranuclear functions. HMGB1 can be passively released by necrotic or damaged cells (2), and in the course of inflammation may also be actively secreted by activated monocytes and macrophages (3). Extracellular HMGB1 acts as a proinflammatory cytokine, induces a proinflammatory response mediated by HMGB1 receptors such as RAGE (receptor for advanced glycation end products) (4), TLR2 (Toll like receptor), TLR4 (5), TREM-1 (Triggering recep-

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tor expressed on myelocytes) resulting in increased production of proinflammatory cytokines (6) and the total amplification of inflammation. Extracellular HMGB1 belongs to DAMPs (Damage associated molecular patterns) and in accordance with the Danger theory, performed by Prof. Matzinger, DAMPs may participate in pathogenesis of diseases in general (7) and also in diagnoses within DPLD. For example, in sarcoidosis heat shock proteins as DAMPs may induce different models of sarcoidosis, depending on the genetic background of the host (8). The exact role of HMGB1 as a danger signal within DPLD is not known so far.

In the light of clinical practice, HMGB1 is considered as a good marker for monitoring inflammation reflecting the fact that compared to other proinflammatory cytokines has a relatively long half-life and wider diagnostic window (9).

Bronchoalveolar lavage is an auxiliary diagnostic method and allows monitoring of the inflammation directly at the site of inflammation, in the lung. Cell subpopulations and various soluble markers such as proteins and glycoproteins may be analysed in BALF samples (10,11). The method is commonly used in differential diagnosis of diffuse parenchymal lung diseases (DPLD). DPLD represent a heterogenous group of diseases comprising more than 200 distinct diagnostic entities. The classification of DPLD is somehow problematic, as in many cases the etiology remains unknown and our knowledge on the pathogenesis of these disorders is limited. DPLD are currently classified according to the consensus of the American Thoracic Society and the European Respiratory Society into four groups: DPLD with a known cause, idiopathic interstitial pneumonias, granulomatous disorders and other forms of DPLD (12,13). The diagnosis of DPLDs is commonly established by HRCT (high resolution computer tomography) and examination of the DLCO (diffusing capacity of the lung for carbon monoxide). However, no specific tests for distinguishing between particular diseases within the DPLD spectrum currently exist, and the diagnosis is made on the basis of results from clinical, radiological, pathological and immunological examinations. A great amount of effort, performed by many study teams, has been put in the finding specific markers for various DPLD, but further studies are necessary for implementation in the routine clinical practice (14,15). We assume that the monitoring of inflammation using the newest markers of inflammation in BAL fluid may lead to a better understanding of the DPLD immunopathogenesis and potentially may be useful in differential diagnosis of pulmonary diseases. Very little information has been found in the literature concerning HMGB1 in BALF in DPLD. Hamada et al. confirmed increased HMGB1levels in idiopathic pulmonary fibrosis and hypersensitive pneumonitis (16). The aim of this study was the analysis of HMGB1 in BALF and correlation of HMGB1 levels with other inflammatory markers and a comparison of HMGB1 level in various diagnoses within DPLD.

Subjects and Methods

Study group

One hundred and thirty subjects with clinical and radiologic signs of DPLD were indicated by physicians to perform a bronchoscopy with BAL to specify the diagnosis. On the basis of further tests, were diagnoses specified. Out of them 15 subjects were Quantiferon TB (tuberculosis) test positive (Interferon-Gamma Release Assay). Subjects with positive Quantiferon TB test were hived off to a separate group. In most cases (13 of 15) QuantiFERON TB-positive subjects had negative bacteriological tests (microscopy and culturing). DPLD were diagnosed on the basis of clinico-radiologic-pathologic criteria in compliance with criteria recommended by the American Thoracic Society (ATS), the European Respiratory Society (ERS), and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG). The study group was divided according to the classification of DPLD used in the joint statement of the American Thoracic Society and the European Respiratory Society: International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias (Table 1) (12,13,17,18).

Ethics statement

The study was approved by the Research Ethics Committee of Faculty of Medicine Comenius University in Bratislava and written informed consent was obtained from all subjects.

Table 1. Study group

Group	Diagnosis	Number (N=) or percentage (%)
DPLD of known cause	Connective tissue disease-assoc.	N=9
	Hypersensitivity pneumonitis	N=7
	Drug induced	N=1
	Radiation induced	N=1
Idiopathic interstitial pneumonias (IIP)	*Chronic fibrosing*	
	Idiopathic pulmonary fibrosis (IPF)	N=19
	Nonspecific interstitial pneumonia (NSIP)	N=1
	Smoking related	
	RB ILD	N=3
	DIP	N=3
	Acute/subacute	
	AIP	N=2
	COP	N=2
Granulomatous lung disorders	Pulmonary sarcoidosis	N=61
	Stage I	14%
	Stage II	64%
	Stage III	7%
	Stage IV	5%
	Löfgren syndrome	13%
Other forms of DPLD	Langerhans' cell histiocytosis	N=1
	Eosinophilic pneumonia	N=5
Quantiferon TB posit.		N=15

AIP - acute interstitial pneumonia, COP - cryptogenic organizing pneumonia, DPLD – diffuse parenchymal lung diseases, RB ILD - respiratory bronchiolitis-interstitial lung disease, DIP - desquamative interstitial pneumonia

BAL procedure

BAL procedure during bronchoscopy was carried out at the Department of Pneumology and Phthisiology, Faculty of Medicine Comenius University in Bratislava. BALF harvesting was performed and the percentage of TREM-1 and TREM-2 positive $CD14^+$ myeloid cells were quantified as previously described (19). Other inflammatory markers (total protein, albumin, IgG, IgA, IgM, C3 complement component, alpha-2 macroglobuline) were measured using commercially available tests in laboratory Medirex, Ltd. HMGB1 level was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) (Shino-test Corporation, Japan /IBL Technology, USA) according to the instructions of the manufacturer.

Statistical Analysis

Data were expressed as mean, standard deviation or median and interquartile range. The one-sample Kolmogorov-Smirnov test was used to determine whether the investigated group followed a normal distribution. According to the results, parametric or non-parametric methods were performed. The different variables were cross-correlated using the Pearson or Spearman test. Either Kruskal-Wallis test (nonparametric ANOVA) or parametric ANOVA was performed to determine the difference and the statistical significance. Statistical analysis of the data was performed using the software program Instat.

Results

HMGB1 and other inflammatory marker correlations

The results of the correlation analysis are presented in Table 2. A positive correlation was found between HMGB1 and total protein, albumin, IgA, IgM and TREM-2 receptor expression. A negative correlation was revealed between HMGB1 and TREM-1 receptor expression. HMGB1 level corre-

Table 2. HMGB1 and other inflammatory marker correlations (all patients)

HMGB1	r	95%CI	P
TN Leu BALF/μl			Ns
MA, Neu, Ly, Eo			Ns
CD3, CD4, CD8, CD4/CD8			Ns
TREM-1 %	-0.3960	-0.5858 – -0.1651	**0.0009 negat.corr.**
TREM-2 %	0.2438	-0.003478 – 0.4630	**0.0468**
Total protein	0.3218	0.1586 – 0.4679	**0.0001**
Albumin	0.2335	0.06409 – 0.3898	**0.0058**
IgG	0.1997		Ns (0.1473)
IgA	0.2215	0.04685 – 0.3831	**0.0110**
IgM	0.1712	-0.0002042 – 0.3328	**0.0439**
C3	0.1684		Ns (0.0521)
Alpha-2 macroglobuline	0.1626		Ns (0.0577)
Number of elevated markers: 1-8 (total protein, albumin, IgG, IgA, IgM, C3, alpha-2 macroglobuline, TREM-2)	0.2709	0.09915 – 0.4271	**0.0017**

TN – total number, MA – macrophages, Neu – neutrophils, Ly – lymphocytes, Eo – eosinophils, TREM % - the percentage of TREM positive cells, CD – cluster of differentiation expression on lymphocytes, C3 – complement component, Ns – not significant

lated with a number of positive markers (P=0.0017) (Table 2).

Comparison of HMGB1 level in various diagnoses

Since only three subgroups of DPLD in our cohort had a sufficient number of subjects to perform statistical analysis, four diagnoses were compared (three DPLD and Quantiferon TB positive subjects): pulmonary sarcoidosis, (N=61), idiopathic pulmonary fibrosis (IPF; N=19), hypersensitivity pneumonitis (N=7) and Quantiferon TB positive subjects (N=15). The highest HMGB1 concentration was found in hypersensitivity pneumonitis and Quantiferon TB positive subjects. A significant difference in HMGB1 level was revealed in Quantiferon TB positive subjects compared with IPF and in hypersensitivity pneumonitis compared with IPF (Table 3).

Comparison of TREM-1 and TREM-2 in various diagnoses

Given the significant correlations between HMGB1 and TREM-1 (negative correlation) and HMGB1 and TREM-2 (positive correlation), we also compared TREM-1 and TREM-2 expression on $CD14^+$ myeloid cells in BALF. The highest percentage of TREM-1 positive $CD14^+$ cells was found in pulmonary sarcoidosis (significantly compared with Quantiferon TB positive subjects) (Table 4).

Table 3. Comparison of HMGB1 level in various diagnoses

	HMGB1	P*
Pulmonary sarcoidosis N=61	Median: **16.8** IQR: 16,85	PS *vs* IPF – Ns PS *vs* TB – Ns PS *vs* HP – Ns
Quantiferon TB positivity N=15	Median: **30.2** IQR: 40,3	TB *vs* IPF – **P<0.05** TB *vs* PS – Ns TB *vs* HP – Ns
Idiopathic pulmonary fibrosis N=19	Median: **8.8** IQR: 14,7	IPF *vs* PS – Ns IPF *vs* TB – **P<0.05** IPF *vs* HP – **P<0.05**
Hypersensitivity pneumonitis N =7	Median: **33.2** IQR: 22,6	HP *vs* PS – Ns HP *vs* TB – Ns HP *vs* IPF – **P<0.05**

*Kruskal-Wallis with post-test (nonparametric ANOVA with Dunn post-test), IQR – interquartile range, *vs* – versus, Ns – not significant

The highest percentage of TREM-2 positive CD14+ cells was revealed in Quantiferon TB positive subjects and in pulmonary sarcoidosis (significantly compared with IPF) (Table 4).

Comparison of neutrophils in various diagnoses

Given the fact that HMGB1 might be a TREM-1 ligand and TREM-1 receptor is constitutively expressed on neutrophils, we compared also percentages of neutrophils in BALF. The highest percentage of neutrophils was found in idiopathic pulmonary fibrosis (statistically significant compared with pulmonary sarcoidosis) followed by hypersensitivity pneumonitis, Quantiferon TB positive subjects and the lowest percentage of neutrophils was found in pulmonary sarcoidosis (Table 5).

Discussion

Diffuse parenchymal lung diseases represent a heterogenous group of diseases comprising more than 200 distinct diagnostic entities with insufficient knowledge of their etiology and pathogenesis, and the lack of diagnostic tests. Analysis of new inflam-

Table 4. Comparison of TREM-1and TREM-2 expression in various diagnoses

	TREM-1	P*	TREM-2	P*
Pulmonary sarcoidosis	Mean (SD): 49.0 SD: 27.0	PS *vs* IPF – Ns PS *vs* TB – **P<0.05** PS *vs* HP – Ns	Mean: 40.0 SD: 22.3	PS *vs* IPF – **P<0.05** PS *vs* TB – Ns PS *vs* HP – Ns
Quantiferon TB posit.	Mean: 20.6 SD: 14.5	TB *vs* IPF – Ns TB *vs* PS – **P<0.05** TB *vs* HP – Ns	Mean: 48.1 SD: 30.2	TB *vs* IPF – **P<0.05** TB *vs* PS – Ns TB *vs* HP – Ns
Idiopathic pulmonary fibrosis	Mean: 40.8 SD: 21.0	IPF *vs* PS – Ns IPF vs TB – Ns IPF *vs* HP – Ns	Mean: 16.2 SD: 6.4	IPF *vs* PS – **P<0.05** IPF *vs* TB – **P<0.05** IPF *vs* HP – Ns
Hypersensitivity pneumonitis	Mean: 25.6 SD: 33.2	HP vs PS – Ns HP vs TB – Ns HP vs IPF – Ns	Mean: 28.4 SD: 16.9	HP *vs* PS – Ns HP *vs* TB – Ns HP *vs* IPF – Ns

P – P value, *parametric ANOVA with Tukey post test, *vs* – versus, SD – standard deviation, Ns – not significant

Table 5. Comparison of the neutrophil percentage in various diagnoses

	Neutrophil %	P*
Pulmonary sarcoidosis	Median: 6.0 IQR: 8	PS *vs* IPF – **P<0.01** PS *vs* TB – Ns PS *vs* HP – Ns
Quantiferon TB posit.	Median: 9.0 IQR: 17	TB *vs* IPF – Ns TB *vs* PS – Ns TB *vs* HP – Ns
Idiopathic pulmonary fibrosis	Median: 14.0 IQR: 18	IPF *vs* PS – Ns IPF *vs* TB – Ns IPF *vs* HP – Ns
Hypersensitivity pneumonitis	Median: 16.0 IQR: 18	HP *vs* PS – Ns HP *vs* TB – Ns HP *vs* IPF – Ns

* Kruskal-Wallis with post-test (nonparametric ANOVA with Dunn post-test), IQR – interquartile range, *vs* – versus, PS – pulmonary sarcoidosis, TB – quantiferon positive patients, IPF – idiopathic pulmonary fibrosis, HP – hypersensitivity pneumonitis, Ns – not significant

matory markers in BALF provides new insights into the immunopathogenesis and may be helpful in differential diagnosis. Extracellular HMGB1 is passively released from damaged necrotic cells or actively produced during inflammation and has proinflammatory activity.

In our study, we evaluated the correlation of HMGB1 with other inflammatory markers in BALF in subjects with DPLD. We found a significant positive correlation between HMGB1 and several inflammatory markers measured in BALF (total protein, albumin, IgA, IgM and percentage of TREM-2 positive myeloid cells). This positive correlation might be a result of HMGB1 proinflammatory function. Macrophages, after coactivation with HMGB1, produce pro-inflammatory cytokines such as TNFα, interleukin-1β, IL-6, IL-8, macrophage inflammatory protein-1α and MIP-2β (20). In addition, HMGB1 induces activation/maturation of dendritic cells (21). In the course of proinflammatory process, the liver produces acute phase proteins and as a result of antigen presentation, mediated by DC, are activated cells of adaptive immunity. Thus, the increase in HMGB1 may indirectly trigger production of various proteins and antibodies.

HMGB1 is called also as late proinflammatory cytokine and given its longer half-life unlike other proinflammatory cytokines, is considered to be a good inflammatory marker. In our study, the number of positive inflammatory markers correlated with HMGB1 level, indicating that HMGB1 could serve as a good marker for the inflammation intensity.

In bronchoalveolar lavage fluid, elevated level of HMGB1 was detected in some pulmonary diseases: pulmonary fibrosis (16), pneumonia (22) and cystic fibrosis (23).

We revealed a negative HMGB1 correlation with the percentage of the TREM-1 positive cells. TREM-1 and TREM-2 are also novel inflammatory markers, potentially useful in differential diagnosis of DPLD according our previous study (19). TREM-1 receptor increases on the surface of myelocytes in the presence of microorganisms and activation of TREM-1 receptor leads to increased production of proinflammatory cytokines (24). The work of El Mezayen et al. suggested that HMGB1 might serve as a potential ligand for TREM-1 (6). The elevated TREM-1 expression on the surface of myelocytes could result in binding of HMGB1 protein, thus reducing the free HMGB1 level and could explain the negative correlation between HMGB1 and TREM-1 (extremely significant).

TREM-2 receptor promotes phagocytosis of bacteria by macrophages (25) and participates in the macrophage fusion and multinucleated cell formation (26). In our cohort, we have found the increased percentage of TREM-1 and TREM-2 positive cells in pulmonary sarcoidosis (consistent with our previous study from 2013 (19)) and increased in TREM-2 positive cells in QuantiFERON TB-positive subjects (Table 4). The common feature of sarcoidosis and tuberculosis is multinucleated giant cell formation, which could explain the increased TREM-2 receptor expression in both diagnoses.

We compared the level of HMGB1 in various diagnoses. The high level of HMGB1 was found in QuantiFERON TB positive subjects (compared with other diagnoses - significantly with IPF). These results are in line with data from Zeng et al., who revealed an elevated HMGB1 level in both serum and sputum in pulmonary tuberculosis (27). Increased HMGB1 may be either the result of cell necrosis and/or raised production of HMGB1 by activated monocytes and macrophages. Mycobacterium tuberculosis can inhibit apoptosis and induce cell necrosis, which is accompanied by nucleus disintegration and the direct release of HMGB1 (28). Grover et al. revealed a direct effect of Mycobacterium tuberculosis on HMGB1 production in monocytes and macrophages in guinea pigs (29). Therefore, the elevated HMGB1 level in tuberculosis may be due to its origin from two sources - passively released by necrotic cells or actively secreted by inflammatory cells. Level of HMGB1 may depend also on uptake by other cells participating in the course of inflammation. Neutrophils can probably participate in HMGB1 uptake since neutrophils constitutively express TREM-1 receptor and undoubtedly play role in TB pathogenesis. Dubaniewicz found increased number and phagocytic activity of neutrophils in TB subjects compared with sarcoidosis and healthy controls (30). We found a slightly higher percentage of neutrophils in QuantiFERON TB positive subjects compared with sarcoidosis (not significant difference). On the other hand, relatively low (compared with sarcoidosis) TREM-1 expression on $CD14^+$ cells (dominated cells in BALF), in the same subjects may cause the low uptake of HMGB1 and results in high levels of free HMGB1 in BALF.

In pulmonary sarcoidosis, HMGB1 values were lower compared with QuantiFERON TB positive subjects and hypersensitivity pneumonitis and higher compared with IPF. An increased TREM-1 expression on $CD14^+$ cells found in pulmonary sarcoidosis (Table 4) can reduce the free HMGB1 by binding the protein to the TREM-1 receptor. This fact can explain the negative correlation between HMGB1 and TREM-1 expression found in our study. Thus, the HMGB1 levels depend on its production and/or uptake by receptors respectively.

Activation of $CD14^+$ cells may play a key role in subsequent inflammatory response. The way they are activated determines the type of inflammation. In the study by Dubaniewicz et al, the authors reported altered expression of FcγR and CR on $CD14^+$ cells and its increased phagocytic activity (31). The increased TREM receptors expression on $CD14^+$ cells provides further knowledge on its pathogenesis.

The significantly higher HMGB1 level was also detected in hypersensitivity pneumonitis (compared with IPF). These results are consistent with the study by Hamada et al., where was found similar levels of HMGB1 in hypersensitivity pneumonitis (16). We found low TREM-1 expression on $CD14^+$ cells in hypersensitivity pneumonitis which might lead to low uptake of free HMGB1 whereupon there was low concentration in BALF.

The lowest HMGB1 level was found in idiopathic pulmonary fibrosis. These values could be elevated compared with healthy subjects as suggested by Hamada et al. Mean and median values in the current study were similar to those referred to Hamada et al (16). A growing body of evidence suggests that, in contrast to other interstitial lung diseases, IPF is a distinct entity in which inflammation is mild/moderate either in early or late disease, and an evolving hypothesis proposes that IPF may result from epithelial microinjuries and abnormal wound healing (32).

Low expression of TREM receptors suggests limited activation of $CD14^+$ cells and agrees with this theory. Relatively low level of HMGB1 in IPF might reflect low release and/or production in course of mild inflammation. In addition, mild/moderate increase in neutrophils might uptake free HMGB1 which can also contribute to low HMGB1 concentration in BALF.

The current study had some limitations: only 130 subjects were enrolled in the study and DPLD is a group of more than 200 diagnoses. We compared only three groups with sufficient number of subjects to statistical analysis and these comparable groups were relatively small. In most cases (13 of 15) QuantiFERON TB-positive subjects were only IGRA test positive and had negative bacteriological tests (microscopy and culturing). Further studies are therefore strongly recommended.

Conclusion

Extracellular HMGB1 in BALF correlated with other inflammatory markers. Interesting findings are correlations with recently discovered receptors TREM-1 and TREM-2. According to recent literature (6), HMGB1 may be a ligand for TREM-1. Our results provide a confirmatory evidence that an increase in TREM-1 expression is accompanied by low HMGB1 level (extremely significant negative correlation between HMGB1 and TREM-1). HMGB1 level varied in different diagnoses; the highest level was found in QuantiFERON TB positive subjects (median 30.2) and hypersensitivity pneumonitis (median 33.2), followed by pulmonary sarcoidosis (median 16.8) and idiopathic pulmonary fibrosis (median 8.8). Analysis of novel inflammatory markers in BALF (HMGB1, TREM-1 and TREM-2) provides new insights into the immunopathogenesis and may be potentially useful in the differential diagnosis of pulmonary diseases. High level of HMGB1 and percentage of TREM-2 positive cells in BALF may suggest mycobacterial infection in subjects in diagnostic process and the need of further specific tests for evidence of mycobacterial infection (IGRA test, Mantoux skin test, PCR mycobacterial DNA, acid-fast bacillus smear and culture).

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