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# Mass spectrometry analysis of redox forms of High-Mobility Group Box-1 Protein in cerebrospinal fluid: initial experience

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## ABSTRACT

**Introduction.** High-mobility group box 1 (HMGB1) is an alarmin with proinflammatory potential determined by redox status of the cysteines at position 23 and 45. It may also play a role as a biomarker in biological fluids. The aim of this study was the identification of different HMGB1 redox forms in cerebrospinal fluid (CSF) obtained from subarachnoid hemorrhage patients.

**Material and Methods.** 6 CSF samples were collected from aneurysmal subarachnoid haemorrhage patients. Commercially available HMGB1 isoforms served as a positive control. Immunoprecipitation and electrophoretic isolation of HMGB1 protein were performed, then both CSF and control were analyzed using mass spectrometry technique. To distinguish between fully reduced (thiol group at C23 and C45) and disulfide (disulfide bond connecting C23 and C45) HMGB1 forms, top-down sequencing of the spectra was performed.

**Results.** Top-down sequencing analysis allowed to distinguish between HMGB1 isoforms only in commercially available standard without preceding immunoprecipitation and electrophoresis. MALDI spectra differ i.e. on the fully reduced HMGB1 spectrum fragmentation occurs before and beyond C22, which is not present on the disulfide HMGB1 spectrum. Analysis of HMGB1 isolated from CSF obtained from subarachnoid hemorrhage patients gave no results.

**Conclusions.** Top-down sequencing enables to distinguish between redox forms of HMGB1. Electrophoresis and tryptic digestion cannot precede mass spectrometry analysis of redox forms of HMGB1 due to the reduction of disulfide bonds during these processes. Preferred method of isolation of HMGB1 for direct analysis using top-down sequencing mustn't include protein digestion or degradation.

**Keywords:** HMGB1 protein; cerebrospinal fluid; MALDI; mass spectrometry; top-down sequencing; redox.

## Introduction

High-mobility group box 1 (HMGB1) protein (also known as amphoterin) was discovered in 1973 and first described as a non-histone chromo-

somal protein and a regulator of gene transcription [1, 2]. Further studies revealed involvement of HMGB1 in pathogenesis of the inflammatory and autoimmune diseases. In 1991 Wang et al. identified HMGB1 as a late mediator of endotoxin lethal-

ity in the mice [3]. What is more, the same article described HMGB1 therapeutic potential as administration of a HMGB1 blocker increased survival rates. Currently, we are aware of HMGB1 involvement in such common diseases as myocardial infarction [4], cerebral ischaemia [5], arthritis [6] and trauma [7]. Both wide expression and diversity of biological actions has led to increase in popularity of HMGB1. Over the last decade number of publications mentioning HMGB1 has increased 3 times (2007: 182 articles; 2017: 633 articles; according to [www.ncbi.nlm.nih.gov/pubmed](http://www.ncbi.nlm.nih.gov/pubmed)). Our own interest focuses on the role of HMGB1 in biological fluids as a biomarker. To extracellular space HMGB1 can be released from the cell interior by both passive (e.g. during necrosis) and active (i.e. secretor vesicles) routes [8]. Outside of a cell HMGB1 binds to the pattern recognition receptors (e.g. Toll-like family) and triggers stereotypical inflammatory reaction associated with increase of interleukin 6 and tumor necrosis factor  $\alpha$  in the neighbour cells [9]. Based on our own experience, HMGB1 can be used as a prognostic biomarker in the subarachnoid haemorrhage (SAH) patients [10]. Nevertheless, in our study we have encountered patients lacking correlation between high HMGB1 level and unfavourable outcome. This might be explained by the differences in redox status of assayed HMGB1. Currently, we are aware that redox status of the three cysteine residues (position 23, 45, 106) determines the extracellular role of HMGB1 [11]. The fully reduced (all-thiol) HMGB1 form induces chemotaxis, the fully oxidized form (sulfonyl HMGB1) has no known function, while form of the intermediate redox status (disulfide HMGB1) induces cytokine production [12]. In the clinical scenario the balance between reactive oxygen species and natural antioxidative enzymes (glutathione peroxidase, catalase, and superoxide dismutase) determines proinflammatory potential of the HMGB1 by influencing its redox status [13, 14]. Unfortunately, the most popular method used to assay HMGB1 level in the biological fluids (enzyme-linked immunosorbent assay) is not capable of distinguishing between forms of HMGB1. Mass spectrometry on the other hand, may be used for HMGB1 redox forms identification [11, 14]. The aim of this study is isolation from the cerebrospinal fluid (CSF) and identification of different isoforms of HMGB1 protein using proteomic tools.

## Material And Methods

### Material

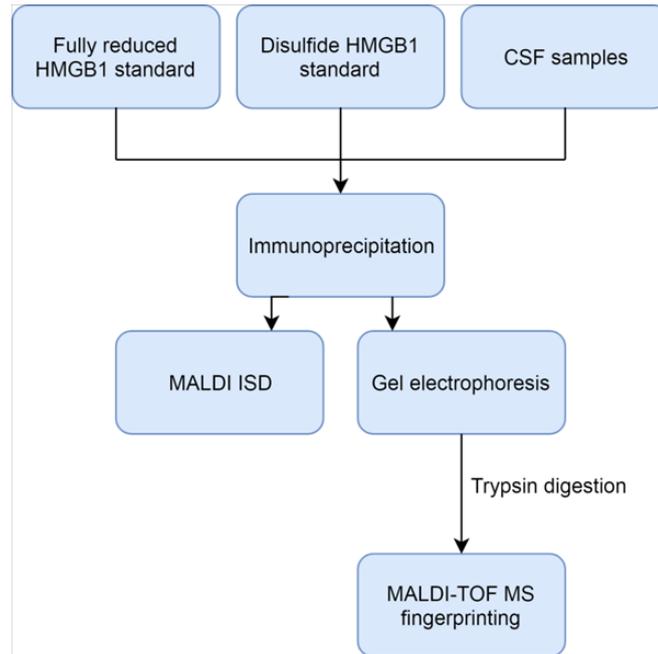
Patients suffering from SAH due to intracranial aneurysm rupture were included in the study. CSF samples were collected on day 0–3, 5 and 10–12 from external ventricular drainage implanted as a treatment for coexisting acute hydrocephalus. After centrifugation samples were stored at -70 Celsius degrees. Patients' treatment outcome was assessed at 6 months. For the analysis we have selected two model patients (no severe comorbidities, typical demographic for SAH) of various treatment outcome (favorable and unfavorable). CSF samples collected at all time points (day 0–3, 5 and 10–12 after aneurysm rupture) were assayed. Commercially available disulfide HMGB1, lipopolysaccharide (LPS) free (HMGBiotech, Milano, Italy) and fully reduced HMGB1, LPS free (HMGBiotech, Milano, Italy) were acquired as standard for comparison with biological samples as well as true positive control.

### Matrix assisted laser desorption/ionization (MALDI) top-down sequencing

Disulfide HMGB1, LPS free and fully reduced HMGB1, LPS free were dissolved in 0.1% trifluoroacetic acid (TFA) in water. Matrix – 1,5-Diaminonaphthalene (1,5-DAN) (Bruker Daltonics, Bremen, Germany) substance was saturated in TA50 solvent (mixture of acetonitrile: 0.1% TFA in water, 50:50). 2  $\mu$ l of matrix solution was mixed with 1  $\mu$ l of sample solution (disulfide HMGB1 and fully reduced HMGB1). 0.5  $\mu$ l of the prepared mixtures were spotted onto the Ground Steel MALDI Target Plate and left to dry at room temperature. Analysis was conducted using MALDI-Time-of-Flight (TOF) mass spectrometer (UltrafleXtreme, Bruker Daltonics, Bremen, Germany) and ion-source decay (ISD) fragmentation technique in the mass range of 1–8 kDa. The mass spectra were externally calibrated using Bovine Serum Albumin (BSA) standard and average mass deviation was below 100 ppm. Acquisition and spectra processing were performed by FlexControl 3.4 software (Bruker Daltonics, Bremen, Germany). Evaluation of the spectra was performed in FlexAnalysis 3.4 software (Bruker Daltonics, Bremen, Germany).

### Immunoprecipitation and analysis of HMGB1 isolated from the cerebrospinal fluid

Full workflow is presented on **Figure 1**. Each assay step is described in detail below.



**Figure 1.** Workflow of immunoprecipitation and analysis of fully reduced HMGB1, disulfide HMGB1 and CSF samples

### *Immunoprecipitation*

Immunoprecipitation procedure was conducted according to protocol provided by Sigma-Aldrich – method A [15]. Briefly, Protein G Sepharose (Sigma Aldrich, St. Louis, Missouri, United States) was washed twice and resuspend with washing buffer (PBS pH 7.4). Agarose conjugate was divided into 90  $\mu$ l aliquots. Next, 10  $\mu$ l of primary antibody Anti-HMGB1 antibody produced in rabbit (Sigma Aldrich, St. Louis, Missouri, United States) was added to each microcentrifuge tube. After incubation and washing procedure, CSF samples and solutions of standard protein (fully reduced and disulfide HMGB1) were added. Each sample was added to two tubes. Agarose conjugate, antibody and samples were incubated overnight in 4°C. After washing procedure each pellet was resuspended in 20  $\mu$ l Laemmli sample buffer (Bio-Rad, Hercules, California, United States) or water and heated at 95°C for 5 min.

### *Gel electrophoresis*

Samples resuspended in Laemmli sample buffer were further separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The 15  $\mu$ l of prepared mixtures were loaded on the 12% Tris-glycine-SDS-PAGE gels. Separation was conducted at 200V for 40 min on a Mini-Protean Tetra Cell (Bio-Rad, Hercules, Cal-

ifornia, United States). The gels were stained with Coomassie Brilliant Blue G250. The 9% acetic acid was used to wash out the background.

### *MALDI-TOF mass spectrometry (MS) fingerprinting*

Stained spots were excised and digested with trypsin according to adapted Shevchenko et al. protocol [16]. Peptides were extracted using 35  $\mu$ l of TA50 solvent. HCCA (alpha-Cyano-4-hydroxycinnamic acid) (Bruker Daltonics, Bremen, Germany) matrix solution was prepared by dissolving 1.4 mg of HCCA in mixture of 85% acetonitrile, 15% water, 0.1% TFA and 1 mM  $\text{NH}_4\text{H}_2\text{PO}_4$ . The 0.5  $\mu$ l of the sample was spotted onto AnchorChip MALDI Target Plate (Bruker Daltonics, Bremen, Germany) and left to dry in the room temperature. Then the 0.5  $\mu$ l of the matrix solution was spotted onto each sample spot and left to dry in the room temperature. Analysis was conducted using MALDI-TOF mass spectrometer (UltrafleXtreme, Bruker Daltonics, Bremen, Germany) in the reflectron mode in the mass range of 700–3500 Da. The mass spectra were externally calibrated using a standard peptide calibration mixture. Acquisition and spectra processing were performed by FlexControl 3.4 software (Bruker Daltonics, Bremen, Germany). Evaluation of the spectra was performed in FlexAnalysis 3.4

software (Bruker Daltonics, Bremen, Germany). Protein searches was conducted using BioTools 3.2 software (Bruker Daltonics, Bremen, Germany) SwissProt database and Mascot 2.4.1 search engine (Matrix Science, London, UK).

### MALDI-ISD analysis

Samples resuspended in water after immunoprecipitation were analyzed using MALDI-ISD method, described earlier in MALDI TOP-DOWN SEQUENCING.

## Results

### MALDI top-down sequencing analysis

MALDI-ISD analysis for disulfide HMGB1 and fully reduced HMGB1 was performed. Top-down sequencing of the spectra was carried out using SwissProt database. From the reduced spectrum high score from the top-down sequencing search giving 'High mobility group protein B1 OS=Homo sapiens GN=HMGB1 PE = 1 SV = 3' was obtained (**Figure 2**). The N-terminus is missing the methionine. According to Swissprot there should be disulfide bond between cysteines at position 23 and 45 in disulfide HMGB1, which is not observed in fully reduced HMGB1 sample (**Figure 3**).

### Immunoprecipitation and analysis of HMGB1 isolated from the cerebrospinal fluid samples

Protein spots on the SDS-PAGE gel appeared near mass 75 kDa and 20–25 kDa (CSF samples) and 25 kDa (HMGB1 standard solutions). Ig gamma protein (75 kDa) and keratin (25 kDa) were identified using MALDI-TOF MS fingerprinting, but HMGB1 was not found. MALDI-ISD analysis of samples after immunoprecipitation procedure gave no results, there was no signal derived from protein.

## Discussion

HMGB1 has been shown to play an important role as a mediator of inflammation. It is also involved in regulation of the immune response [17]. As HMGB1 seems to be implicated in diseases characterized by cell damage and death [18], it may serve as an indicator of SAH. Depending on appearance in different cellular compartments and redox status of HMGB1, its functions may vary. The aim of this study was identification of different HMGB1 redox forms using advanced mass spectrometry techniques. Obtained results confirm possibility of distinguishing between fully reduced and disulfide forms. In order to determine differences between redox forms with MALDI,

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1 MGKGDPPKPR GKSSYAFFV QTCREEHKKK HPDASVNFSE FSKKCSERWK
51 TMSAKEKGF EDMAKADKAR YEREMKTYIP PKGETKFKFK DPNAPKRPPS
101 AFFLFCSEYR PKIKGEHPGL SIGDVAKKLG EMWNNTAADD KQPYEKKA
151 LKEKYEKIDIA AYRAKGPDA AKKGVVKA EKSKKKKEEED EDEDEDEEE
201 EDEDEDEEE DDDDE

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**Figure 2.** Top-down sequencing search obtained from the fully reduced HMGB1 spectrum

Amino acid modifications				
	Feature key	Position(s)		Description
T	MOD_RES	184	184	{ECO:0000250 UniProtKB:P10103}. N6-acetyllysine.
T	MOD_RES	185	185	{ECO:0000250 UniProtKB:P10103}. N6-acetyllysine.
T	DISULFID	23	45	In disulfide HMGB1. {ECO:0000250 UniProtKB:P63159}.
T	VARIANT	11	11	G -> R (in gastric-carcinoma cell line). {ECO:0000269 PubMed:9036861}. /FTId=VAR_046451.
T	VARIANT	149	149	A -> E (in gastric-carcinoma cell line). {ECO:0000269 PubMed:9036861}.

**Figure 3.** SwissProt database result, which confirmed that the cysteines at position 23 and 45 should be bonded in disulfide HMGB1 sample

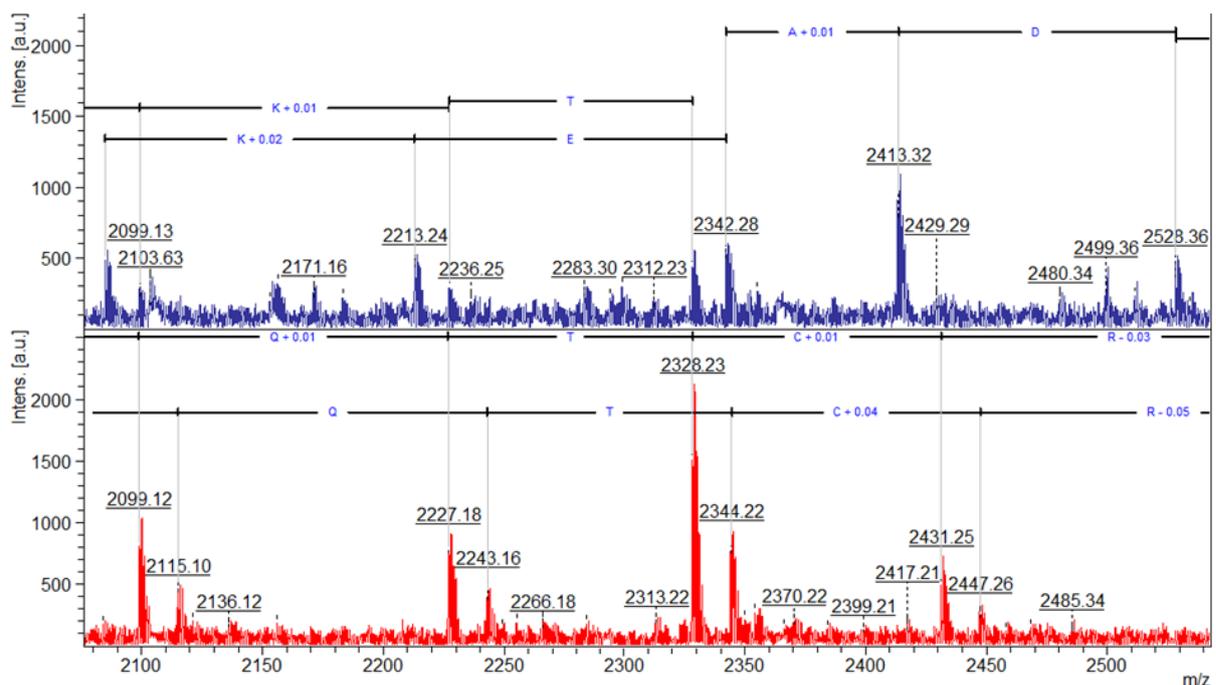
disulfide HMGB1 and fully reduced HMGB1 were analyzed. MALDI-MSD analysis was performed to obtain fragmentation of the study proteins. Then top-down sequencing of the spectra was performed. In fully reduced HMGB1 cysteines at the positions 23 and 45 do not contain a disulfide bond. If the same sequence was assigned to disulfide HMGB1 sample, the sequence stops at position 21, which is right before the cysteine (**Figure 4**). However, there are quite some signals in the mass range > 2431 Da, which are unexplained. For a real verification of the disulfide link between positions 23 and 45 a signal at 5128 Da is needed, which then includes both oxidized cysteines.

In the next step of the study we made an attempt to isolate HMGB1 from the CSF of SAH patients by immunoprecipitation method, gel electrophoresis and trypsin digestion in order to identify its redox form using MALDI-TOF fingerprinting technique. Immunoprecipitation, a type of affinity purification, is one of the most widely used immunochemical techniques and it can be used to determine presence of proteins in biological material [19]. Following immunoprecipitation MALDI-MSD analysis was carried out. Despite the fact, that initial analysis of pure standard HMGB1 by MALDI-MSD was successful, after immunoprecipitation we were not able to identify HMGB1 in both

CSF samples as well as standard. In the next step electrophoresis, digestion and MALDI-TOF MS fingerprinting analysis was carried out for remaining samples. Unfortunately, although keratin (25kDa) and Ig gamma protein (75kDa) were identified, we obtained no signal derived from HMGB1 in MALDI-TOF MS fingerprint method. In order to improve immunoprecipitation step we have used different antibody concentrations i.e. 1.25 µg/ml, 12.5 µg/ml and 100 µg/ml, but the results remained negative. Possible reasons of failure on immunoprecipitation step might be: degradation of HMGB1 antibody, inadequate time of incubation, inadequate washing solution or non-specific binding to Protein G. Nevertheless, since reduction of disulfide bonds is necessary step for tryptic digestion, it would be impossible to distinguish two forms of HMGB1 protein using this method. The preferred method of isolation of HMGB1 should allow for directly analysis using top-down MALDI-MSD strategy, without protein digestion or degradation.

## Perspectives

As HMGB1 has been shown to play an important role in inflammation and cell damage processes, it may act as prognostic marker in the



**Figure 4.** MALDI mass spectra of the fully reduced HMGB1 (blue) and disulfide HMGB1 (red). On the first spectrum (blue) fragmentation occurs before and beyond C22, which is not present on the second spectrum (red)

SAH patients. In further studies there is a need for optimization or development of new methods allowing isolation of HMGB1 from CSF and distinguishing between fully reduced and oxidized forms. We expect, that quantitative assay of different redox status forms would improve prognostic accuracy of HMGB1 as well as broaden our knowledge regarding pathophysiology of SAH.

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### Conflict of interest statement

The authors declare no conflict of interest.

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