

High Levels of HMGB1 in Plasma May Be due to *Ex Vivo* Cell Necrosis

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Abstract. *Background: High mobility group box 1 protein (HMGB1) is considered an important biological marker during inflammation and malignancies. Here, we evaluated sample handling and effects of ex vivo necrosis on HMGB1 levels. Materials and Methods: Plasma samples were obtained from healthy volunteers (n=14) simulating the standard laboratory conditions, overnight incubations and harsh treatment. HMGB1 levels were evaluated by ELISA or western blot. Additionally, levels of hemoglobin, hemolysis index and lactate dehydrogenase were measured. Results: Plasma levels of HMGB1 were 9-fold increased in samples stored overnight at room temperature, as compared to those processed directly. The rapid centrifugation prevented the increase of HMGB1 in stored samples. Hemoglobin, hemolysis index and lactate dehydrogenase concentrations showed significant correlations with HMGB1 levels. Conclusion: Handling of blood samples is important for the accurate estimation of systemic HMGB1. We propose that all samples with high HMGB1 concentrations should be evaluated for markers of ex vivo necrosis.*

High-mobility group box 1 protein (HMGB1) is a nonhistone nuclear protein that performs a dual function (1). Intracellularly, HMGB1 binds DNA, regulates transcription, and determines chromosomal architecture. Extracellularly, HMGB1 activates the innate immune system and mediates a wide range of physiological and pathological responses through interactions with the receptor for advanced glycation end-products (RAGE) as well as with Toll-like receptor (TLR) 4. The protein has been proposed as being the key factor, signaling cell damage, coordinating inflammation and tissue

repair (1). The molecule has also been implicated in cancer conditions, stimulating cellular proliferation, invasiveness and angiogenesis (2). Thus, overexpression of HMGB1 has been reported in cancer cells (3) and the protein has been identified as an important component of distorted tumor microenvironment (4). Elevated HMGB1 levels correlating with clinical outcome has been reported in a variety of clinical conditions with acute/chronic inflammation and malignancies (1). However, the plasma HMGB1 concentration does not always correlate with survival and biological parameters. Even if differences could be partly attributed to different methods of HMGB1 detection in biological fluids, the differences are also noticeable in the studies that concordantly use ELISA for plasma HMGB1 evaluation. In an article published in a recent issue of Anticancer Research (5) Lehner *et al.*, evaluate HMGB1 immunoassay, emphasizing on the importance of sample handling and its influence on the HMGB1 levels, as determined by ELISA. Even if we agree with the overall conclusion of the authors, we are missing a stronger emphasis on the link between the high levels of HMGB1 and sample quality and the proposals on how to evaluate the “false-high HMGB1 levels” in clinical samples due to *ex vivo* cell damage.

We have been involved in HMGB1 research in several years and we welcomed the Shino-Test HMGB1 immunoassay in 2006. However, using the HMGB1 assay, we noticed how important sample handling is, as well as the existence of a limitation of the assay when applied to the stored samples or samples in which processing was impossible to supervise. The latter happens often during collaborative research. That is why we carried out a small evaluation study to determine the surrogate of sample quality and the handling procedure that affect the least HMGB1 measurement.

Materials and Methods

Clinical material. We collected blood samples by venipuncture from 14 healthy individuals (vacutainer EDTA-tubes for plasma and serum tubes without anticoagulant) performed by the same experienced nurse in the outpatient clinic at the Department of Infectious diseases, Huddinge, Sweden. Tubes without anticoagulant

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were left on the bench to coagulate for 15 min (room temperature [RT]). Plasma and serum samples were obtained after centrifugation at 1600 \times g for 15 min. Vacutainer tubes from eight patients were handled in different conditions as to compare the effect of different preparation techniques. The single tubes were handled as follows: i) direct plasma preparation (within one hour); ii) incubation overnight at 4°C with or without direct centrifugation at 1600 \times g for 15 min; iii) incubation overnight at room temperature with or without direct centrifugation at 1600 \times g /15 minutes; iv) vortexing for 20 s at low speed; v) rapid freeze-thaw cycle at -20°C versus 37°C (warm bath).

The presumed conditions were chosen to 1) mimic the situation of harsh transportation (vortex); 2) mimic the conditions of the overnight storage at the laboratory; 3) measure the high amount of HMGB1 due to necrosis. Additionally, plasma and serum samples were obtained from six individuals where the plasma samples were collected according to points i) and iii).

Plasma analysis. HMGB1 levels were determined by commercial ELISA (Shino-Test Corporation, Japan), according to the manufacturer's instructions. Western-blot analysis was used to confirm the presence of HMGB1 in plasma. The procedure was carried out as previously described (6), utilizing a rabbit anti-HMGB1 polyclonal antibody (ABCAM, UK). Additionally in plasma, we assessed the grade of cell damage by measuring the concentration of free plasma hemoglobin (Hb), lactate dehydrogenase (LD) and hemolysis index at the clinical routine laboratory of Karolinska University Hospital, Huddinge, Sweden.

Statistics. For all comparisons, means (or medians) were calculated. Differences between the treatments were elucidated by the Kruskal-Wallis test, followed by the Dunn's post-test. Correlation analyses were performed using the Spearman method. The GraphPad Prism Software, (San Diego California, USA) was used for all tests.

Results

We found low HMGB1 levels at the baseline in all 14 patients (median 0.99 ng/ml; range 0.51-3.5), as expected in a healthy population. Interestingly, we noticed more than 9-fold increase of HMGB1 levels (Figure 1a) if the blood samples were left overnight (o/n) on the lab bench (median 9.46 ng/ml; range 2.6-17.0; $p<0.05$) and slight increase if the samples were stored at 4°C (median 1.67 ng/ml; range 1.1-2.8). This difference was reduced if the blood samples left o/n in room temperature were directly centrifuged after collection (median 1.07 ng/ml; range 0.61-1.49) or centrifuged and stored overnight at 4°C (median 0.95 ng/ml; range 0.59-1.27) concordantly with results of Lehner J. *et al* (5). The increase of HMGB1 levels were observed if the tubes were vortexed for 20-30 s (median 5.1 ng/ml; range 3.98-7.09). The freeze/thaw cycle of the whole blood resulted in significant release of HMGB1 (median 156 ng/ml; range 29-263). Interestingly, the differences between the HMGB1 levels in serum and plasma samples were negligible when compared during direct plasma/serum preparations and overnight

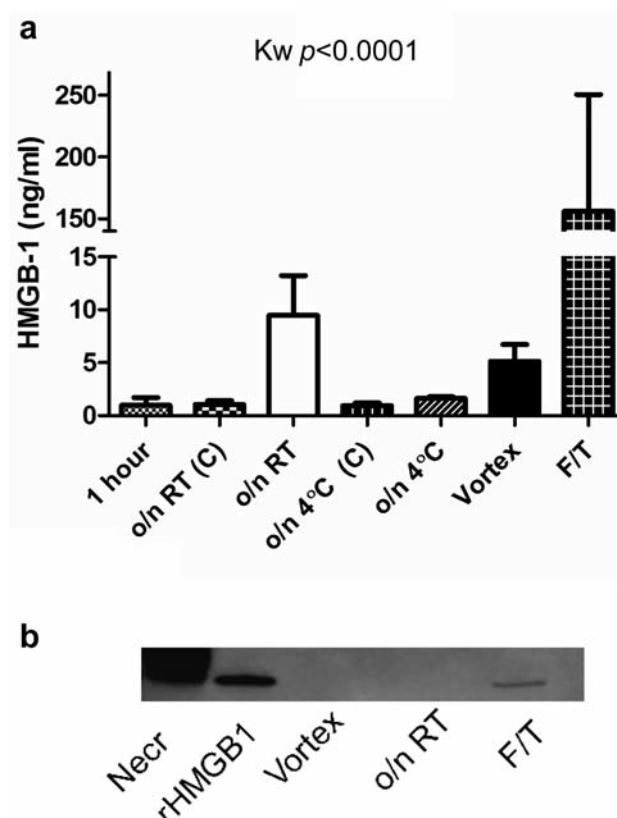


Figure 1. a. Levels of HMGB1 in plasma samples exposed to different preanalytical conditions. The conditions included analysis: after 1 hour, overnight (o/n) storage at 4°C or room temperature (RT), vortexing for 20-30 seconds, one cycle of freeze/thawing (F/T). Data are presented as median, interquartile range. p-Value refers to Kruskal-Wallis analysis with Graph Prism Software. (C) -indicates the centrifugation step. b. Western blot analysis of HMGB1 in plasma samples obtained after vortexing (vortex), overnight incubation (o/n) and freeze/thaw (F/T) cycle. Necrotic extract from PBMCs and recombinant HMGB1 (100 ng; R&D systems, MN, USA) served as a control.

incubations (data not shown). To exclude the possibility of cell damage products interference with the readout of the ELISA, the western blot analysis was conducted on plasma samples. This experiment clearly revealed single bands corresponding to the molecular weight of HMGB1 in the freeze/thawed sample (Figure 1b). We performed the analysis of cell damage /hemolytic markers (lactate dehydrogenase and hemolysis index) as well as hemoglobin in tested samples (n=55). We noticed an increase of all markers in the samples with high amount of HMGB1, especially in those who underwent harsh treatment. Thus, hemolysis index ($r=0.46$; $p=0.0004$), Hb ($r=0.44$; $p=0.002$) and LD ($r=0.43$; $p=0.0009$) showed positive correlation with HMGB1.

Discussion

The data presented in this short report illustrate the importance of preanalytical sample handling, when estimating HMGB1 levels. Thus we present that both “harsh” sample management as well as blood storage conditions could substantially influence the outcome of HMGB1 evaluation.

Our results are in line with the data presented by Lehner J. *et al* (5). Their observation that the levels of HMGB1 were elevated in serum samples as compared to plasma is unexpected, as HMGB1 is a “sticky protein” and lower levels of HMGB1 in sera as compared to plasma were reported, although the results may be prone to individual diversity (7). It could be speculated that the serum separation tubes used by the authors could influence the results (8). We have also found that centrifugation of the blood samples was beneficial for the protection from cell necrosis, although the overnight incubation in the fridge condition was as good. The latter, possibly mirrors the preservation of cell structure in conditions of cold (9).

The harsh treatment (mild vortexing) as well as freeze thawing of the blood samples has resulted in the detection of considerable amounts of HMGB1 in the plasma. This should bring special attention to the retrospective studies that use older samples or samples where the collection procedure is not standardized. The results obtained under these conditions should be, thus, cautiously interpreted. Unfortunately, we do not provide a direct answer which will help to reject the *ex vivo* hemolysed samples from the presumed analysis. Although both LD, Hb and hemolysis index correlated to HMGB1, the correlations are moderate, and more extensive studies are required to achieve a suitable algorithm. Moreover, utilization of LD and Hb levels is rather limited in clinical conditions where the elevated levels of these clinical markers are part of the disease.

The potential sources of *ex vivo* HMGB1 are all nucleated cells in the blood or platelets. The red blood cells (RBC) unlikely contribute to plasma levels as these cells lack nucleus and the presence of HMGB1 was not confirmed by western blot analysis of erythrocytes’ cell lysates (10). Additionally, elevated levels of HMGB1 in RBC packages could be decreased due to leukoreduction and did not increase with 42 days of storage which suggests a different source than RBC (11).

In conclusion, we show that handling of blood samples is important to properly estimate the systemic HMGB1 levels and *ex vivo* hemolysis/cell necrosis may considerably contribute to elevated levels of HMGB1. Our observations are certainly applicable to other systemic measurements of

intracellular biomarkers that could be released during cell damage. We suggest that after the performed analysis of HMGB1 levels in plasma, all outliers’ samples should be evaluated for the markers of *ex vivo* hemolysis/cell necrosis.

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