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Human HMGB1 does not induce eryptosis in vitro

Maryna Tkachenko[1,2], Anatolii Onishchenko[2], Dmytro Butov[3], Tetyana Butova[2], Anton Tkachenko[2]

[1]Department of Internal Medicine No. 2, Clinical Immunology and Allergology named after academician L.T. Malaya, Kharkiv National Medical University, Kharkiv, Ukraine
[2]Research Institute of Experimental and Clinical Medicine, Kharkiv National Medical University, Kharkiv, Ukraine
[3]Department of Phthisiology and Pulmonology,Kharkiv National Medical University, Kharkiv, Ukraine

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Corresponding author:
Anton Tkachenko.
E-mail: as.tkachenko@knmu.edu.ua;
ORCID: 0000-0002-1029-1636

Abstract

Aim: To study the ability of human high mobility group box protein 1 (HMGB1) to induce eryptosis *in vitro*.

Material and methods: Blood collected from six healthy volunteers was incubated with HMGB1 (0-0.2-1-5 ng per ml). Eryptosis of red blood cells was assessed by Annexin V staining and 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) staining by flow cytometry. The forward scatter (FSC) fluorescence was used to evaluate the morphology of red blood cells.

Results: Exposure of erythrocytes to HMGB1 did not affect the morphology of erythrocytes, evidenced by no changes in the percentage of cells with small volume, i.e. shrunken cells, and erythrocytes with large volume, i.e. enlarged cells. HMGB1 had no impact on phosphatidylserine externalization, which is confirmed by the absence of statistically significant changes in the amount of phosphatidylserine-displaying cells and the mean fluorescence intensity (MFI) values of Annexin V-FITC in cells exposed to different concentrations of HMGB1. Furthermore, H2DCFDA staining revealed that the HMGB1 did not induce oxidative stress.

Conclusion: HMGB1 does not promote eryptosis of human erythrocytes at concentrations of up to 5 ng/ml.

Key words: high mobility group box protein 1, erythrocytes, inflammation, cell death

Introduction

Human high mobility group box protein 1 (HMGB1) is a highly abundant evolutionary conserved nuclear protein composed of 215 amino acid residues with a molecular weight of approximately 30 kD [1]. It has attracted a lot of attention, since it can be secreted by cells or released from dead cells and act extracellularly as an alarmin or damage-associated molecular pattern (DAMP) [2,3]. As for its intracellular functions, HMGB1 is a non-histone, chromatin-binding protein, which is involved in transcription regulation and DNA repair [1,4]. However, nowadays effects of extracellular HMGB1 are under extensive research due to its ability to regulate inflammation. In particular, HMGB1 secreted from viable cells or cells, which underwent apoptosis, necrosis, netosis, pyroptosis, and necroptosis, can induce the inflammatory response [5-9]. It has been reported that extracellular HMGB1 is a ligand for many receptors, including the innate immunity ones, but its pro-inflammatory effects are mainly mediated by binding to toll-like receptor-4 (TLR4) and receptor of advanced glycation end products (RAGE) [10]. Both TLR4 and RAGE signaling culminates in nuclear factor-kB (NF-kB) activation and generation of pro-inflammatory cytokines [11].

It is important to mention that HMGB1 can undergo some post-translational modifications, such as oxidation, acetylation, methylation, phosphorylation, ubiquitination, glycosylation and ADP-ribosylation, which can affect its effects on cells [1]. Inside the nucleus, HMGB1 is fully reduced and its oxidation is tightly regulated both under normal and pathological conditions [12]. Reduced and oxidized forms of HMGB1 differ in their effects on inflammation, which suggests the importance of HMGB1 redox state and its regulation [13,14].

In addition to inflammation, HMGB1 is involved in regulation of cell death. It is mainly considered to be an inhibitor of apoptosis and necrosis [15]. However, some evidence is provided that HMGB1 can induce apoptosis in tumor cells [16]. Moreover, this DAMP can promote autophagy, necroptosis, and pyroptosis [16-18]. Moreover, HMGB1 is reported to regulate the crosstalk between autophagy and apoptosis in inflammation [19]. Thus, HMGB1 is a multi-faceted protein involved in regulation of inflammation and cell death. Its effects on cells are pleiotropic and context-dependent. Despite the fact that erythrocytes don't have nucleus and unlikely release HMGB1, its role in red cell biology is of huge interest. No data are available on the ability of human HMGB1 to influence eryptosis, which is a suicidal cell death of red blood cells.

The study was designed to analyze the impact of human HMGB1 on eryptosis *in vitro*.

Material and methods

Subjects and incubation conditions

Blood samples were collected from six conditionally healthy male volunteers aged 24-29 years in K2EDTA Vacutainers (IMPROVACUTER Evacuated EDTA K2 Spray Dried PET Tubes, Guangzhou, China). All volunteers enrolled for the study signed an informed consent. Exclusion criteria included the presence of acute or chronic inflammatory diseases, hypertension, endocrine diseases, intake of steroid hormones for the last 3 months, obesity (BMI over 30), and fever. Blood aliquots of 5 µl were incubated with human HMGB1 (0-0.2-1-5 ng per ml, n = 6) purchased from Elabscience (Houston, TX, USA) in 500 µl RPMI-1640 medium with stable glutamine (Biowest, France) and 5% fetal bovine serum (BioWhittaker®, Lonza, Belgium) for 24h in sterile conditions. The sample size was calculated using G*Power software (Germany).

Following incubation, erythrocytes were obtained after washing the samples twice with phosphate buffer saline (PBS). The obtained erythrocyte suspensions were used for Annexin V staining and 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) staining.

The research was conducted in accordance with the Declaration of Helsinki and adhered to Good Clinical Practice guidelines. The design was approved by the Ethics and Bioethics Committee of Kharkiv National Medical University (Kharkiv, Ukraine; minutes No 5 dated September 17, 2019). The study was performed in December 2021.

Annexin V staining

Erythrocyte suspensions obtained from blood exposed to HMGB1 were stained with Annexin V-FITC purchased from Becton Dickinson (USA). Erythrocytes were resuspended in 100 µl 1x Annexin-binding buffer (Becton Dickinson, USA) after washing and then 5 µl Annexin V-FITC was added. Cells were incubated for 15 minutes. Then 400 µl 1x Annexin-binding buffer was added to each tube. The fluorescence of Annexin V-FITC was detected by BD FACSCanto™ II flow cytometer. Samples treated with hydrogen peroxide (0.1 mM) were used as positive controls. Negative controls included erythrocyte suspensions treated with no Annexin V-FITC [20, 21].

H2DCFDA staining

To analyze reactive oxygen species (ROS) generation, erythrocyte suspensions were stained with H2DCFDA. According to the staining protocol [20, 21], washed red blood cells were resuspended in 100 µl PBS. Thereafter, stock solution of H2DCFDA (5 mM) was added, so that the working solutions had 5µM H2DCFDA. Suspensions were incubated in the dark for 30 minutes, and then washed in PBS to remove the extracellularly located dye and resuspended in 500 µl PBS for acquiring the fluorescence of dichlorofluorescein (DCF), produced in cells from H2DCFDA when interacting with intracellular ROS. The fluorescence of DCF was registered by BD FACSCanto™ II flow cytometer. Excitation wavelength was 488 nm, while the emission wavelength was 525 nm for both Annexin V-FITC and DCF.

Eryptosis and cell morphology indices

FlowJo™ (v10, BD Biosciences, USA) and BD FACSDiva™ software (Becton Dickinson, USA) were used to process initial files of fluorescence acquisition. To analyze the impact of HMGB1 on eryptosis, five parameters were compared. The morphology of erythrocytes exposed to HMGB1 was assessed by comparing the percentage of cells with small volume (FSC-low) and large volume (FSC-high) [22]. To analyze the cell membrane scrambling, the percentage of Annexin V-positive cells and the mean fluorescence intensity (MFI) values of Annexin V-FITC were detected. The MFI values of DCF characterized ROS production in erythrocytes.

Statistical analysis

The Kruskal-Wallis and *post-hoc* Dunn's tests were used to statistically process the data obtained in this study. All numerical values are shown in figures as the median (Me) and interquartile range (IQR; 25%–75%). p values above 0.05 indicated no statistical significance. Graph Pad Prism 5.0 (USA) software was used to process the results.

Results

Analysis of forward scatter (FSC) histograms of erythrocyte suspensions exposed to various concentrations of HMGB1 allowed identifying three populations of cells, i.e. the cells with normal volume, small (FSC-low) and large (FSC-high) volumes, respectively (Figure 1a). The percentage of cells with small and large volumes, i.e. shrunken or enlarged erythrocytes,

Figure 1 - Forward scatter (FSC) intensity analysis for determination of red blood cell volume. Representative forward scatter (FSC) histograms of erythrocytes treated with HMGB1 (0-0.2-1-5 ng per ml) for 24 h. The populations of shrunken (FSC-low) and enlarged (FSC-high) erythrocytes are shown (panel a). The impact of HMGB1 (0-0.2-1-5 ng / ml) on the morphology of erythrocytes. The percentages of FSC-low (shrunken) and FSC-high (enlarged) cells were found to be comparable in four groups of samples (n=6).

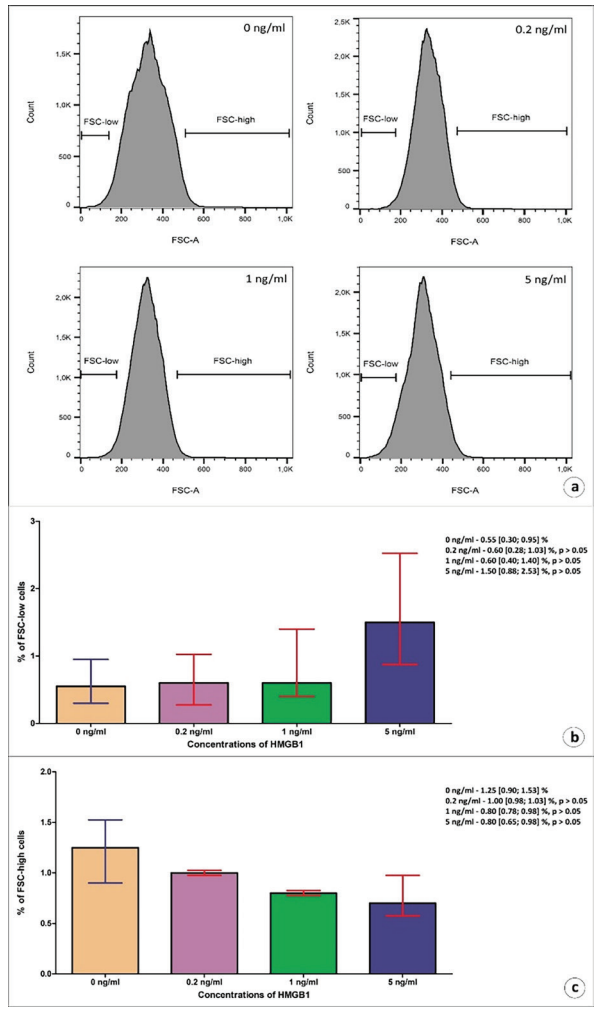
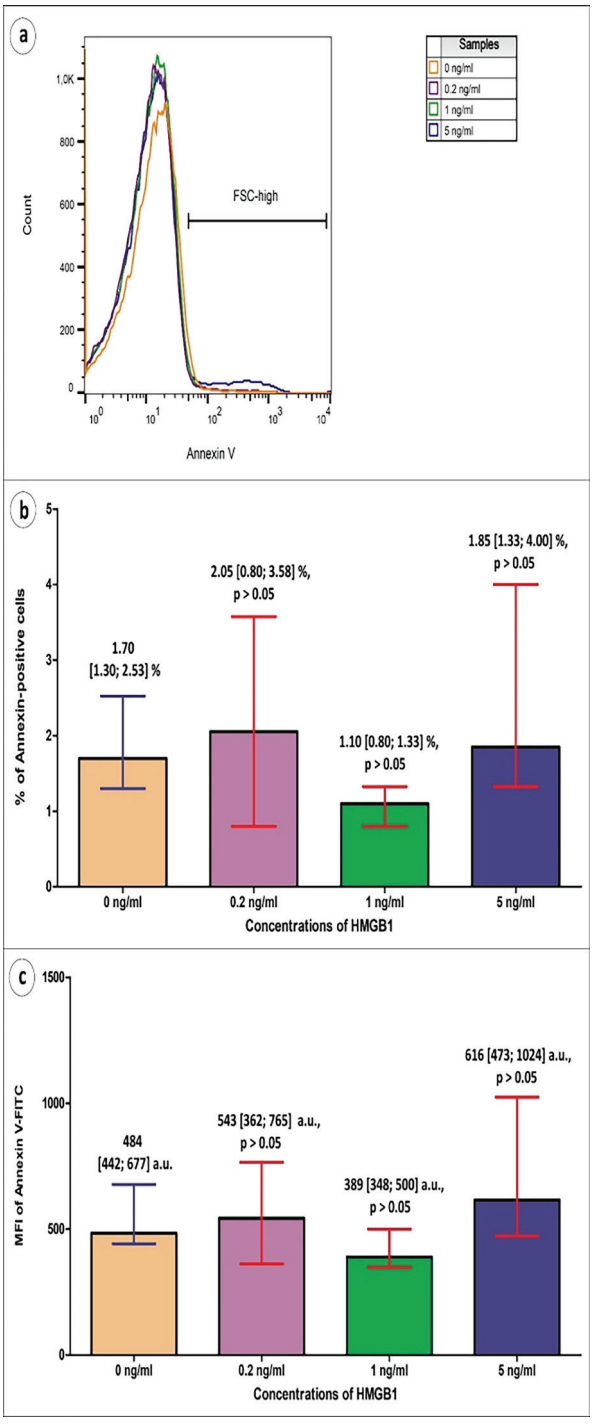


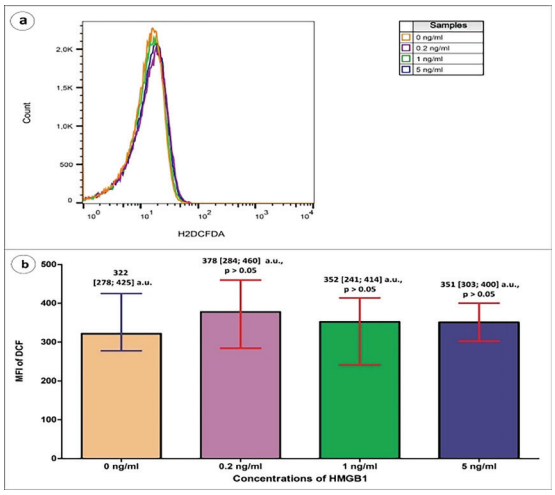
Figure 2 - Phosphatidylserine externalization in erythrocytes exposed to HMGB1 (0-0.2-1-5 ng per ml) for 24 h. Representative side scatter (SSC)/FL1 histograms are shown (panel a). Phosphatidylserine externalization was assessed quantitatively by comparing the percentage of Annexin V-positive cells (panel b) and the mean fluorescence intensity (MFI) values of Annexin V-FITC (panel c). Both eryptosis indices differed statistically insignificantly.



respectively, were compared between 4 groups of samples exposed to different concentrations of HMGB1. No statistically significant differences were found in these parameters (Figure 1b,c). Thus, incubation of blood with HMGB1 did not cause the changes in the morphology of red blood cells.

Annexin V staining was used to analyze the rate of phosphatidylserine externalization in cell membrane of cells, which is a hallmark of eryptosis. Two parameters that characterize the cell membrane scrambling were analyzed, namely the percentage of cells with externalized phosphatidylserine (Annexin V-positive cells) and the MFI values of Annexin V-FITC.

Figure 3 - HMGB1 did not promote ROS production in erythrocytes. H2DCFDA staining of red blood cells incubated with HMGB1 (0-0.2-1-5 ng per ml) for 24 h. Representative side scatter (SSC)/FL1 histograms are demonstrated (panel a). Generation of reactive oxygen species (ROS) was estimated by comparing the mean fluorescence intensity (MFI) values of dichlorofluorescein (panel b).



The latter reflects the degree of phosphatidylserine translocation to the outer leaflet of cell membranes. Both parameters were observed to be unaffected (Figure 2) indicating no changes in phosphatidylserine externalization in response to HMGB1.

Another marker of eryptosis is ROS overgeneration, which can be analyzed by H2DCFDA staining. No statistically significant changes in the MFI values of dichlorofluorescein, which is produced inside the cells from H2DCFDA upon the interaction with ROS, were revealed after incubation with different concentrations of HMGB1 (Figure 3).

Discussion

In this study, the impact of human HMGB1 on eryptosis of red blood cells was assessed in a complex way by analyzing the morphology of erythrocytes, the cell membrane scrambling and oxidative stress development. Eryptosis is a form of suicidal, programmed cell death of red blood cells characterized by certain morphological changes, including cell shrinkage [23]. This is a protective mechanism used to eliminate dysfunctional cells from the bloodstream. Multiple triggers of eryptosis have been described, including energy deficiency, osmolar damage to erythrocytes, action of xenobiotics and ROS [24]. It is important to note that the hallmarks of eryptosis are cell shrinkage, phosphatidylserine externalization, i.e. translocation of this phospholipid from the inner layer of cell membrane to the outer leaflet, oxidative stress and calcium ion entry [25]. Thus, these parameters can be used as reliable markers of eryptosis.

HMGB1 is known to regulate multiple cell death modes. However, its impact on eryptosis is not studied. The average HMGB1 levels in serum of healthy individuals vary from 0 to 1.3 ng/ml (on average 0.2-0.5 ng/ml) and can increase over 20-fold in case of various pathological conditions [26-28]. Thus, circulating erythrocytes can contact high concentrations of HMGB1.

There is accumulating evidence that the HMGB1–TLR4 and HMGB1–RAGE axes mediate apoptosis induced by HMGB1 [29, 30]. However, despite the reports on the ability of erythrocytes to modulate innate immunity and express some innate immunity receptors, including TLRs [31], the role of

innate immunity pathways in erythrocytes is extremely limited. We believe that this could be the reason behind the inability of HMGB1 to induce eryptosis. In addition, it has been reported that the impact of HMGB1 is context-sensitive and depends on crosstalks between many factors, which limits the results of *in vitro* studies [15]. Nevertheless, our findings indicate that HMGB1 does not induce eryptosis of human erythrocytes despite the widely recognized ability of this alarmin to regulate apoptosis of different cells.

The study has some limitations. In particular, this research was performed on a relatively small sample size. In addition, not all eryptotic indices that can elucidate underlying mechanisms, including intracellular calcium ion levels and ceramide abundance, were analyzed.

Conclusion

Human HMGB1 does not induce eryptosis of erythrocytes at concentrations below 5 ng/ml. Nor HMGB1 triggers ROS overproduction and oxidative stress. This study contributes to covering the knowledge gap in understanding the impact of HMGB1 on cell death and survival. Future studies are necessary to reveal the influence of higher levels of HMGB1 on eryptosis to figure out the role of HMGB1 in eryptosis regulation under pathological circumstances.

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