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Biochemistry

High Mobility Group B1 Protein Secretion in Polarized Macrophages

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High mobility group B1 proteins are actively secreted in the extracellular space by various immune and non-immune cells. In response to multiple stimuli, intracellular HMGB1 induces proliferation, inflammation, angiogenesis and inhibits host antitumor immunity. The behavior of HMGB1 depends on its binding to various membrane receptors, including the receptor for advanced glycation end-products (RAGE). Our work aimed to see whether the levels of intracellular and extracellular HMGB1 and the expression of RAGE changed during macrophage polarization. Our results have shown that intracellular HMGB1 increased in the M2 phenotype as a result of polarization. Besides, the expression of the membrane RAGE was reduced in polarized macrophages. We propose that the increased HMGB1 in the M2 phenotype is probably due to its lysosomal store, expecting an additional signal for excretion from the cell. These data suggest that HMGB1-RAGE complex intracellular delay occurs in M2 macrophages, which in turn leads to retention of inflammatory response. © *2021 Bull. Georg. Natl. Acad. Sci.*

Macrophage polarization, HMGB1, RAGE, inflammation

Macrophages are functionally heterogeneous cells and play an essential role in protecting the host organism and maintaining tissue homeostasis. According to the stimuli in the microenvironment, they are polarized into two main groups: classically activated macrophages or M1 and alternatively activated macrophages – M2. It has been experimentally observed that many signaling molecules are involved in the process of polarization of macrophages and change it in different ways. Phenotypic transformation of macrophages has also been observed in many pathological conditions [1].

High mobility group B1 proteins (HMGB1) are non-histone chromosomal proteins with quite various functions inside the cell and in the extracellular space. HMGB1 is involved in the maintenance of nucleosomal structure in the nucleus and the regulation of gene transcription. It is also actively secreted in the extracellular space by various immune and non-immune cells, such as macrophages, monocytes, neutrophils, dendritic cells, and natural killer cells. In response to

multiple stimuli, intracellular HMGB1 induces proliferation, inflammation, energy metabolism, angiogenesis and inhibits host antitumor immunity [2].

Studies have shown that HMGB1 present in monocytes and macrophages undergoes strong acetylation after its activation by LPS and localizes the protein in the cytosol [3]. Cytosolic HMGB1 is then concentrated in secretory lysosomes and released after the corresponding signal. Outside the cell, this protein acts as a cytokine and is a late mediator of inflammation. Two different ways of secreting HMGB1 into the extracellular environment are described: the first is its active secretion from activated macrophages and monocytes and its passive secretion from necrotic and damaged cells [4].

The behavior of HMGB1 depends on its binding to various membrane receptors, including RAGE (receptor for advanced glycation endproducts) [5]. Adhesion of the receptor and HMGB1 results in the activation of many kinases, including ERK1 / ERK2, p38MAP kinase, and JNK kinase [6], and also become a cyclic-AMP-binding protein (cAMP response element-binding protein [7]. The HMGB1 signal to RAGE activates the NF-κB pathway and the signal transduction signal to the JNK, p38, and ERK/MAP kinase pathways [8]. The HMGB1 / RAGE bond is directly responsible for expressing adhesive molecules such as VCAM-1 and ICAM-1. It induces the secretion of chemokines-CXCL12 [9], which in turn forms a heterocomplex with HMGB1.

RAGE is involved in many other HMGB1-dependent signaling pathways, such as pathways that control thrombosis, dendritic T cell migration to lymph nodes, T cell activation and angiogenesis, Brain damage after a heart attack [10].

Given the above, the purpose of this study was to see whether the levels of intracellular and extracellular HMGB1 and RAGE changed during macrophage polarization.

Methods

Cell culture. Mouse RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC) and cultured in plastic cell culture flasks (Greiner Bio One), at 37 °C under 5% CO2/95% air in Dulbecco's Modification of Eagle's Medium (DMEM; ATCC) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma), 100 U/ml penicillin (Gibco® by Life Technologies) and 100 µg/ml streptomycin (Gibco® by Life Technologies). RAW 264.7 macrophages were used between passage 5 and 15.

Macrophage polarization. RAW 264.7 cells were treated with cytokines to induce either M1 or M2 cell state. For M1 polarization, cells were treated for 24 hours with 20 ng/mL of interferongamma (IFN-γ) (ab123747; Abcam, Cambridge, UK) and 100 ng/mL of lipopolysaccharide (LPS) (L2880-100MG; Sigma-Aldrich Co.); for M2 polarization, in the presence of 20 ng/mL of IL-4 (ab191628; Abcam) and 10 ng/mL of IL-10 (BMS347; eBioscience, Vienna, Austria). Cell viability was assessed by staining the cells with Trypan blue (#1450021; Bio-Rad, Hercules, CA, USA) using an automated Cell Counter TC 20TM (Bio-Rad). The cells were then harvested using Cell Scraper (C6106-100EA; Greiner Bio One, Frickenhausen Germany), and the harvested cells were maintained in growth medium and used for further analysis.

Western blot analysis. Following incubation, cells were removed from the dishes, washed with PBS, and homogenized in ice-cold lysis buffer containing 100 mM NaCl, 1 mM EDTA, 0.5% Triton X100, 50 mM Tris-HCl, pH 7.4, and protease inhibitors 1 mM PMSF, 5 mg/mL aprotinin, 5 mg/mL pepstatin A, and 5 mg/mL leupeptin. Lysates were incubated at 4°C for 30 minutes followed by centrifugation at 13,000× g for 15 minutes. Fifty micrograms of proteins were denatured at 90°C for 5 minutes, separated by SDS-PAGE on 15% gels. After electrophoresis, the

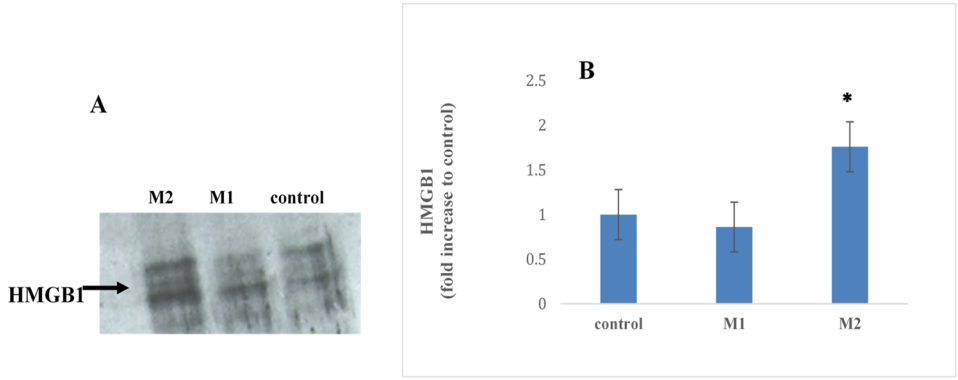

Fig. 1. Expression of HMGB1 proteins in lysates of control and polarized macrophages. A) Immunoblotting of proteins labeled with anti-HMGB1 antibody; B) Densitometric data of the protein blot. Data are given as mean ± SD. * P <0.05, relative to control.

proteins were transferred onto a nitrocellulose membrane (UltraCruzTM Nitrocellulose Pure Transfer Membrane; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After blocking with 5% BSA and 0.05% Tween 20 in Tris-HCl buffered saline, the membranes were incubated with the corresponding primary antibodies (antiRAGE [ab172473; Abcam] and anti-HMGB1 [sc-26351; Santa-Cruz Biotechnology]), and immuno-reactivity was detected by enhanced chemiluminescence autoradiography (ECL kit, sc-2048; Santa-Cruz Biotechnology). Protein concentrations were determined using a BCA protein assay kit (sc-202389; Santa Cruz Biotechnology).

Statistical analysis. All data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Scheffe's post hoc comparison test. P<0.05 was considered statistically significant.

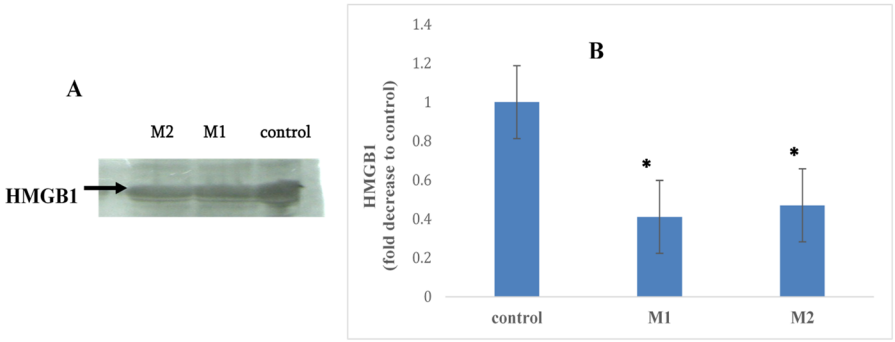

Fig. 2. HMGB1 protein secretion in the extracellular area of control and polarized macrophages. A) Immunoblotting of proteins labeled with anti-HMGB1 antibody; B) Densitometric data of the protein block. Data are given as mean ± SD. * P <0.05, relative to control.

Results and Discussions

There is considerable evidence that HMGB1 proteins play an essential role in inflammatory diseases, including septic shock, acute pneumonia, rheumatoid arthritis, acute coronary syndrome, and others. HMGB1 proteins are somewhat self-generating immune activators, and they have multiple functions in regulating immunity and inflammatory processes. Thus, we hypothesized

that the levels of HMGB1 might be changed in the macrophage polarization process.

In our experiments, we used RAW 264.7 macrophage cell cultures. We polarized these cells, namely to obtain the M1 phenotype, we added LPS / INFy and the M2 phenotype - IL-4 / IL-10 to the cells. Polarization occurred at 24 ° C. After 24 h of incubation, the cells were centrifuged at low speed (1500 rpm), and the supernatants were collected for further analysis. Concentrated supernatants were subjected to electrophoresis on a polyacrylamide gel and then transferred to the nitrocellulose membrane. The cells were treated by lysis buffer, and in lysates, the expression of HMGB1 proteins was determined. The changes in the expression of HMGB1 proteins in the lysates of the control (non-polarized) macrophages, M1 and M2 were analyzed. It was found that as a result of polarization, the expression of HMGB1 proteins increased compared to the control in the M2 phenotype, with a decrease in the M1 phenotype, although this decrease was not statistically significant (Fig. 1):

In a subsequent series of experiments, we determined the extracellular amount of HMGB1, secreted from macrophages. It was found that protein secretion was significantly reduced (almost 2-fold) in both the M1 and M2 phenotypes compared to controls (Fig.2), and this reduction was found to be statistically significant. An interesting aspect of our data is that the expression of HMGB1 proteins in M2 phenotype lysates

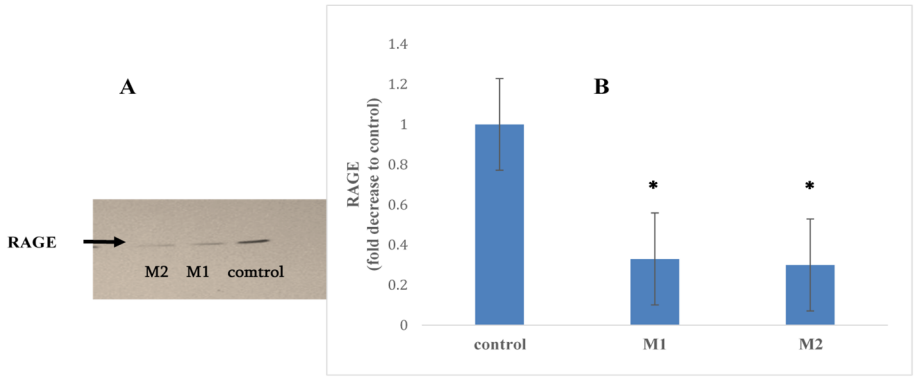

Fig. 3. Expression of RAGE into membrane fractions of control and polarized macrophages. A) analysis of anti-RAGE antibody-labeled protein immunoblotting; B) Densitometric data of the protein block. Data are given as mean ± SD. * P <0.05, relative to control.

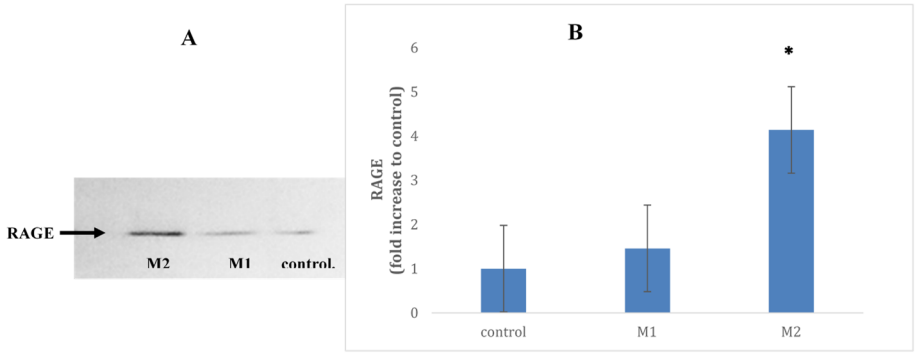

Fig. 4. Expression of RAGE in lysates of control and polarized macrophages. A) analysis of anti-RAGE antibody-labeled protein immunoblotting; B) Densitometric data of the protein block. Data are given as mean ± SD. * P <0.05, relative to control.

increases. At the same time, the amount of protein extracted from the cell decreases compared to controls.

HMGB1 proteins, as mentioned above, are multifunctional proteins and trigger inflammatory responses by binding to the RAGE receptor. The HMGB1-RAGE complex is involved in many pathologies; thus, we determined the expression of RAGE receptors, both in cell membrane fractions and in cell lysates. The degree of expression of this protein in membrane fractions was found to be much higher in the control cells than in the polarized phenotypes. A small, statistically insignificant difference was observed between the M1 and M2 phenotypes (Fig.3)

We determined the number of RAGE receptors in cell lysates. It was found that the amount of protein in cell lysates increased in the M2 phenotype compared to the control and M1 phenotype. A slight increase of RAGE secretion was observed in the M1 phenotype, although this elevation was not statistically significant (Fig.4).

Several studies have shown that dynamin-dependent endocytosis of HMGB1 is triggered due to HMGB1- RAGE interaction, which initiates cellular and molecular processes. These include activation of cathepsin B and release from degraded lysosomes, leading to the formation of a pyroptosome and activation of caspase-1, which promotes HMGB1-induced pyroptosis. Our results have shown that intracellular HMGB1 increased in the M2 phenotype as a result of polarization. The reduced secretion in control cells may be explained by the fact that HMGB1 accumulates in the lysosomes in the cell and requires a special signal to exit the lysosomal supply. Therefore, the increased HMGB1 in the M2 phenotype is probably due to its lysosomal store, expecting an additional signal for excretion from the cell. Interestingly, the expression of the membrane RAGE was reduced in polarized macrophages. It was suggested that both HMGB1 and RAGE intracellular delays occur in M2 macrophages, resulting in inflammatory response retention.

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ბიოქიმია

მაღალი მობილობის ჯგუფ B1-ის ცილების ექსპრესია პოლარიზებულ მაკროფაგებში

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მაღალი მობილობის ჯგუფ B1-ის ცილები (მმჯბ1) აქტიურად გამოიყოფა უჯრედშორის სივრცეში სხვადასხვა იმუნური და არაიმუნური უჯრედების მიერ. ანთებითი სტიმულის საპასუხოდ, უჯრედშიდა მმჯბ1 იწვევს პროლიფერაციას, ანთებას, ანგიოგენეზს და აფერხებს მასპინძლის იმუნიტეტს სიმსივნის წინააღმდეგ. მმჯბ1-ის ქცევა დამოკიდებულია მის ურთიერთობაზე სხვადასხვა მემბრანულ რეცეპტორებთან, მათ შორის, გლიკაციის საბოლოო პროდუქტების რეცეპტორთან. ჩვენი სამუშაოს მიზანს წარმოადგენდა იმის გარკვევა, იცვლებოდა თუ არა უჯრედშიდა და უჯრედგარე მმჯბ1 დონე და გლიკაციის საბოლოო პროდუქტების რეცეპტორის ექსპრესია მაკროფაგების პოლარიზაციის დროს. ჩვენმა შედეგებმა აჩვენა, რომ უჯრედშიდა მმჯბ1-ის დონე პოლარიზაციის შედეგად გაიზარდა M2 ფენოტიპში, რაც, შესაძლებელია, განპირობებულია მისი ლიზოსომური მარაგის გაზრდით, რომელიც უჯრედიდან გამოყოფისათვის დამატებით სიგნალს ელოდება. საინტერესოა, რომ პარალელურად, პოლარიზებულ მაკროფაგებში მემბრანული გლიკაციის საბოლოო პროდუქტების რეცეპტორის ექსპრესია შემცირდა. ამ ყველაფრის გათვალისწინებით, ჩვენ ვარაუდობთ, რომ მმჯბ1-გლიკაციის საბოლოო პროდუქტების რეცეპტორის კომპლექსის უჯრედშიდა შეკავება ხდება M2 მაკროფაგებში, რაც, თავის მხრივ, იწვევს ანთებითი რეაქციის შეკავებას.

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