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THE EFFECT OF HMGB1 PROTEIN AND ITS TRUNCATED FORM ON THE EXPRESSION OF RAGE VARIANTS IN BREAST CANCER CELLS

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Abstract

HMGB1 protein is a DNA binding nuclear protein. Its properties to bind different non-B DNA conformations and also to bend linear DNA implicate the protein in many essential cellular processes as DNA replication, repair, transcription, remodelling, etc. HMGB1 plays important role outside the cell as it is passively released from necrotic cells and actively from apoptotic ones and binds its specific receptor for advanced glycation end products RAGE. HMGB1/RAGE interactin is implicated in various diseases including cancer. Different soluble RAGE forms were reported whose functional role is to serve as a decoy for the ligands and in this way to block the signalling pathway. How the ligands regulate the production of RAGE variants is a subject of great scientific interest. We studied the effect of HMGB1 and its truncated form lacking the C-terminus on the expression of full-length (flRAGE) and soluble RAGE (sRAGE) in breast cancer cell lines: MCF7 represents a hormone dependent cancer with better prognosis and MDA-MB-231 – hormone independent with substantial invasive capacity. HMGB1 stimulates the total RAGE expression in both breast cancer cells but in MCF7 the ratio changes and is in favour of the membrane fraction. The absence of the C-tail of HMGB1 provokes comparable changes in RAGE production in MCF7 cells as the whole HMGB1 molecule. In MDA-MB-231cell line the total amount of RAGE was slightly affected but it is entirely represented by the membrane form and the soluble one is in negligible amounts.

Key words: HMGB1, RAGE, breast cancer cells

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Introduction. High Mobility Group B1 (HMGB1) protein is a DNA binding nuclear protein consisting of 215 amino acids that are organized in two L shaped HMG boxes and a nonstructural acidic C tail. It is considered an "architectural factor" of chromatin as a result of its ability to recognize and bind with high affinity to different distorted DNA structures [1,2]. The properties of HMGB1 to bind different non-B DNA conformations and also to bend linear DNA implicate the protein in many essential cellular processes as DNA replication, repair, transcription, remodeling, etc. [3]. It is interesting to note that some of HMGB1 protein's activity is regulated by the acidic C-terminal [4,5]. It turned out that HMGB1 plays important role outside the cell too [3]. It is passively released from necrotic cells and actively from apoptotic ones [6,7]. In the extracellular medium HMGB1 binds its specific receptor for advanced glycation end products (RAGE). RAGE activation plays a role in various diseases, including cancer, chronic inflammation, sepsis, arthritis, diabetic nephropathy, atherosclerosis [8,9]. The receptor is composed of three immunoglobulin-like regions: one "V"-type domain responsible for ligand binding, two "C"-type-domains, a short transmembrane domain and a cytoplasmic tail critical for intracellular signalling. It is considered that one of the effects of HMGB1/RAGE interaction is the activation of the proinflammatory transcription factor NF-κB [10]. In turn RAGE expression is induced by NF-κB and results in upregulation of the receptor. Various RAGE truncated forms were identified lacking the transmembrane region and the cytosolic tail. They are considered a result either of alternative splicing [11,12], or products of the activity of metalloproteases [13]. The functional role of the soluble form of RAGE is gaining more and more interest as it may serve as a decoy for the ligands and in this way to block the signalling pathway. The importance of this pathway in vivo is suggested by the observation that blockade of HMGB1 and RAGE suppresses tumour growth and metastasis [14]. The ratio between the soluble and membrane form of RAGE can serve as a marker for positive or negative disease development. How the ligands regulate the production of RAGE variants is a subject of great scientific interest. We studied the effect of HMGB1 and its truncated form lacking the C-terminus on the expression of full-length (flRAGE) and soluble RAGE (sRAGE) in breast cancer cell lines.

Materials and methods. ***Polymerase chain reaction* (*PCR*).** DNA constructs for full-length recombinant HMGB1, its truncated tail-less molecule (HMGB1ΔC),) were prepared by PCR amplification of cDNA encoding full-length rat HMGB1 (lib.N 961, RZPD). The primers used introduce EcoR1 and XhoI cloning sites for the full length HMGB1 forward 5′-TGCACTGGAATTCATGGG CAAAGGAGATCC-3′ and reverse 5′-CAGTGCACTCGAGTTATTCATCATCA TCATCTTC-3′ and for the truncated form HMGB1ΔC forward 5′-TGCACTGG AATTCATGGGCAAAGGAGATCC-3′ and reverse 5′-CTTCTTTTTCTTGCTT TTTTCAGCCTTG-3′, respectively.

Expression of recombinant proteins. The PCR products were treated with the restriction enzymes EcoR1 and XhoI, cloned in an expression vector

pET28a+ and expressed in modified *Escherichia coli* BL21 Poly Lys S. His-tagged protein samples were purified on a HIS-Select HF Nickel Affinity gel (Sigma). The purity of all protein preparations was checked by polyacrylamide gel electrophoresis containing sodium dodecyl sulphate (SDS-PAGE).

Cell cultures. Human MDA-MB-231 and MCF-7 breast cancer cell lines (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher) supplemented with 10% fetal bovine serum (Thermo Fisher), 5% L-glutamine (PAA) and 1% penicillin/streptomycin (PAA).

Western blot analysis. Total protein lysates were obtained by lysing the cells with RIPA buffer supplemented with proteinase inhibitor cocktail (Roche) and quantified by Bradford assay (Bio-Rad). Two hundred micrograms of each sample were denatured in sample buffer (50 mM Tris HCl pH 6.8; 2% SDS; 10% glycerol; 1% β-mercaptoethanol; 12.5 mM EDTA, 0.02% bromphenol blue), subjected to a 12% SDS-PAGE transferred to nitrocellulose membranes incubated ON at 4 °C with the appropriate antibodies: anti-RAGE (AB9714, Merck Millipore 1:1000), anti-β-actin (Thermo Fisher; 1:2000). Proteins were visualized using LI-COR Odyssey IR imaging system with appropriate IRDye-labelled secondary antibodies (LI-COR Biosciences). The relative RAGE levels were quantified using Image J software and normalized to β-actin.

Results. Two breast cancer cell lines were chosen for our study: MCF7 represents a hormone dependent cancer with better prognosis and MDA-MB-231 – hormone independent, demonstrating substantial invasive capacity. Recombinant full length HMGB1 and the truncated form lacking the C-terminus (HMGB1ΔC) were generated by PCR and purified as described in Materials and methods section. The electrophoresis profile of both proteins are presented in Fig. 1.

The cells at their confluent state were incubated with 200 ng final protein concentration for 7 h, lysed, run on SDS polyacrylamide gel, and transferred to nitrocellulose membrane. The RAGE fractions were visualized by immune reaction with monoclonal anti-RAGE antibody. Two bands with molecular weight of 55 kDa and 38 kDa that correspond to the full-length RAGE (flRAGE) and its soluble form sRAGE, respectively, [15] were normalized to actin as a reference and plotted. Untreated cell lines were used as a control. The results for MCF7 are presented in Fig. 2.

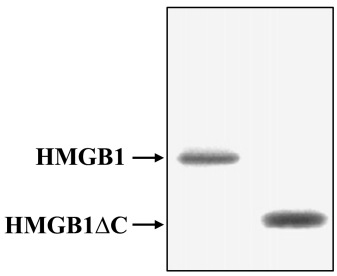

Fig. 1. Purified rHMGB1 and rHMGB1ΔC are subjected to 15% SDS PAGE. The respective protein fractions are marked with arrows

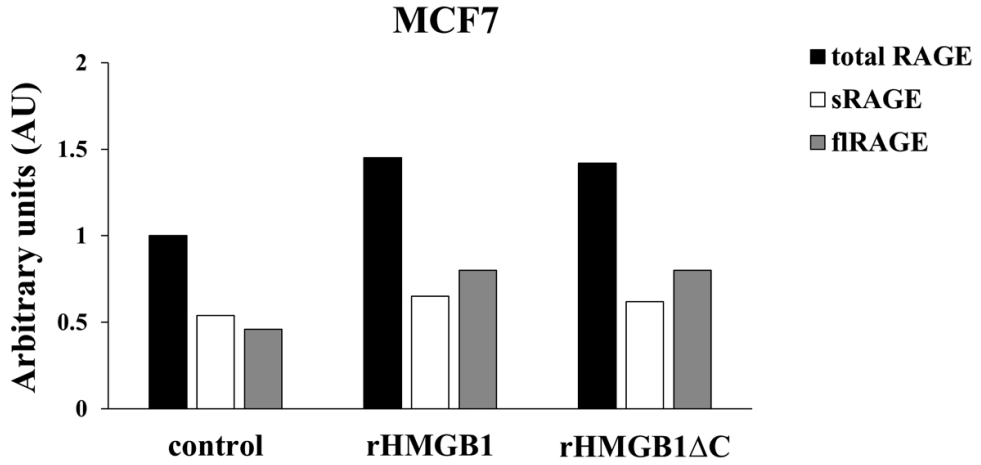

Fig. 2. The signals obtained with specific anti-RAGE antibody for breast cancer MCF7 cell culture extracts were visualized with Odyssey Infrared Imaging System (LI-COR) and the relative flRAGE and sRAGE levels were quantified using Image J software, normalized to β-actin and plotted

It is interesting to note that the ratio of flRAGE and sRAGE in the controls differs: in MCF7 cells the soluble form prevails while in MDA-MB-231 cell line the membrane form of the receptor is approximately twice the soluble one (see Fig. 3). The effect of HMGB1 on RAGE production in MDA-MB-231 results in 1.5 times increase in total amount of RAGE and the ratio between flRAGE and sRAGE is preserved.

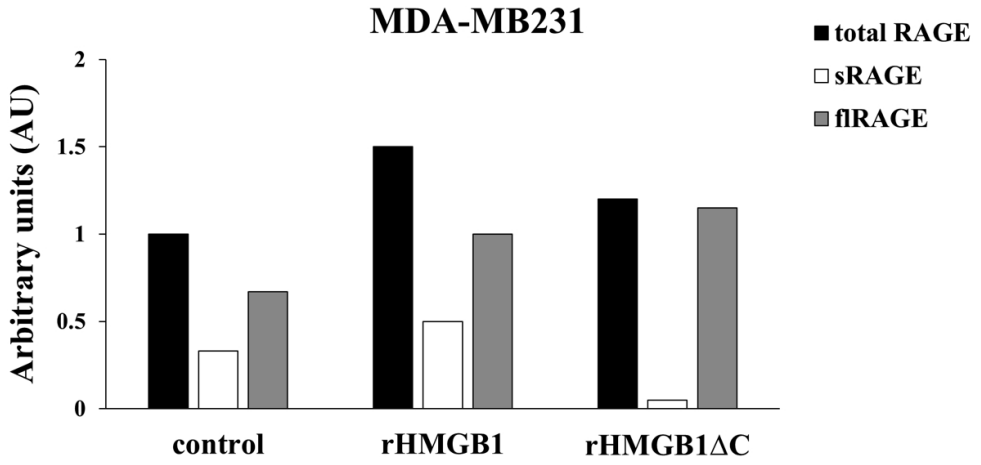

Fig. 3. The signals obtained with specific anti-RAGE antibody for breast cancer MDA-MB-231 cell culture extracts were visualized with Odyssey Infrared Imaging System (LI-COR) and the relative flRAGE and sRAGE levels were quantified using Image J software, normalized to β-actin and plotted

HMGB1 also stimulates the total RAGE expression approximately 1.5 times in MCF7 but in this case the ratio changes compared to the untreated cells and is in favour of the membrane fraction. Quite interesting results were obtained when the breast cancer cells were treated with the truncated form HMGB1ΔC. The absence of the C-tail of HMGB1 provokes comparable changes in RAGE production in MCF7 cells as the whole HMGB1 molecule. The case for MDA-MB-231cell line is absolutely different. The total amount of RAGE was slightly affected but it is entirely represented by the membrane form and the soluble one is in negligible amounts (compared with the control MDA-MB-231cells).

Discussion. As mentioned in the introduction the upregulation of RAGE is tightly related to the development of numerous diseases as chronic inflammation and progression of diabetic vascular complications, cardiovascular disease (CVD), and cancer progression and metastasis [16]. The signals are generated by binding of different ligands, one of them HMGB1, and transmitted to the cell by the membrane form of the receptor and especially by its cytosolic domain. The existence of soluble RAGE is important as it may serve as a trap for the ligands and hamper their interaction with full-length RAGE thus preventing the intracellular signalling. In this respect the ratio between flRAGE and sRAGE may serve as a biomarker for positive or negative disease development outcome [17]. Our results support such a hypothesis; in MCF7 breast cancer that is less invasive and with better prognosis the ratio is in favour of the soluble RAGE while in MDA-MB-231 type that is characteristic for its metastatic potential, the membrane form dominates. One approach for understanding the role of RAGE is to study the effect of its specific ligands as HMGB1. In our experiments we included also the truncated form lacking the C-terminus. Such a form of HMGB1 was found to exist in vivo as a result from cleavage of the acidic tail by specific protease [18]. In both breast cancer cell lines HMGB1 stimulates the expression of total RAGE but changing the ratio in favour of the membrane form only in MCF7 cells. This finding suggests that in the presence of HMGB1 molecule as a ligand the features of less invasive cancer cells start to resemble the characteristics of those with invasive potential. The results obtained with HMGB1ΔC demonstrated that the truncated form had striking effect on the RAGE expression ratio in MDA-MB-231 cells stimulating the synthesis only of the membrane receptor. A COOH-terminal motif in HMGB1 consisting of amino acids 150–183 (the end of the B-box and its linker to the acidic tail) has been identified as responsible for RAGE binding [19]. A reasonable explanation of our finding is that the elimination of the C-tail (last 30 amino acids after the 186 aa) exposes the binding motif of HMGB1 and induces more effective binding to the receptor. Another hypothesis is that the reported conformation changes due to C terminal deletion [20] is more favourable for ligand/receptor interaction. Other possibilities should also be considered in the future investigations, for example the ability of HMGB1 and HMGB1ΔC to form a complex with other pro-inflammatory molecules in different cancer cell lines.

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