Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences
Tome 75, No 6, 2022

BIOLOGY
Molecular biology

THE RATIO OF RAGE ISOFORMS IS AFFECTED BY HMGB1 AND ITS TRUNCATED FORM ONLY IN A549 BUT NOT IN H1299 LUNG CANCER CELL LINES

Jordana Todorova, Maria Petrova, Evdokia Pasheva, Iva Ugrinova#

Received on June 26, 2020
Presented by I. Ivanov, Member of BAS, on September 29, 2020

Abstract

The Receptor of Advanced Glycated End products (RAGE) could exist in two forms, a membrane bound and a soluble one. RAGE is highly expressed during embryonic development and decreases in all tissues except the lung in adult organisms. Lung RAGE mediates lung cancer, pulmonary fibrosis, chronic obstructive pulmonary disease (COPD), cystic fibrosis, etc. The lung cancers, among the most invasive of tumours, are reported to express low levels of RAGE. A specific RAGE ligand is the nuclear protein HMGB1. We examined the effect of HMGB1 and its truncated form lacking the C-tail (HMGB1ΔC) on RAGE expression in NSCLC cancer cell lines with different invasive capacity: A549 with better prognosis and H1299 with negative outcome. In A549 upon addition of ligands two important results are observed: (i) total RAGE expression is augmented 1.5 times and 1.7 times for HMGB1 and HMGB1ΔC, respectively, and (ii), a full-length membrane variant (flRAGE) is observed and in the presence of HMGB1ΔC its expression is comparable with the soluble one. In control H1299 lung cancer cells the dominant form of RAGE was the flRAGE while sRAGE represents one third of the whole amount of the receptor. HMGB1 and HMGB1ΔC do not affect the total RAGE amount and the ratio between flRAGE and sRAGE. In the case of lung cancer, the higher amount of RAGE is related to better prognoses and less metastatic ability which means

#Corresponding author.
The work is supported by DN01/10 16.12.2016 grant from Bulgarian National Science Fund.
DOI:10.7546/CRABS.2022.06.06

that the stimulation of RAGE expression by HMGB1 and HMGB1ΔC in A549 is considered a positive tendency. In the case of the more aggressive lung cancer H1299 both ligands do not change the RAGE behaviour and in this way do not affect the invasive potential of the cancer cells.

Key words: HMGB1, HMGB1ΔC, RAGE, lung cancer cells

Introduction. The Receptor of Advanced Glycated End products (RAGE) belongs to the immunoglobulin superfamily and could exist in two forms, a full-length membrane bound (flRAGE) and a soluble one (sRAGE) [1]. The full-length receptor consists of several functional domains: an extracellular one that is responsible for binding RAGE ligands, a hydrophobic transmembrane domain, and a cytoplasmic part that transmits the intracellular signalling. The soluble RAGE is a product of alternative splicing events or proteolytic cleavage of flRAGE by matrix metalloproteinases. It lacks the transmembrane and the cytosolic parts and contains only the extracellular domain [2–4]. RAGE is highly expressed during embryonic development, but its production decreases in all tissues except the lung in adult organisms [5]. It was reported that in lung RAGE mediates the allergic airway inflammation (AAI), lung cancer, pulmonary fibrosis, chronic obstructive pulmonary disease (COPD), cystic fibrosis, etc. [6]. The receptor can bind a variety of ligands and is known as a pattern recognition receptor [7]. One of the specific RAGE ligands turned out to be the nuclear protein HMGB1. In the nucleus the protein plays important role in many essential cellular processes as repair, replication, remodelling etc., as some of them are modulated by the C-terminus of HMGB1 [8]. HMGB1 protein can also be passively released from damaged cells as a proinflammatory alarmin [9]. Macrophages and dendritic cells can actively secrete HMGB1 molecule [10]. In many tumours as prostate, colon and gastric tumour the blockade of RAGE reduces tumour cell growth and metastases. The lung cancers, among the most invasive of tumours, are reported to express low levels of RAGE [11]. Reduced levels of RAGE have been observed in non-small cell lung carcinoma (NSCLC) compared with the normal lung [12]. It is very interesting to stress on the fact that overexpression of RAGE in lung cancer cells without additional presence of HMGB1 does not mediate an increased tumour growth in athymic mice [13]. The piling evidence in the literature for the role of HMGB1/RAGE ligand receptor pair in lung cancer development motivated us to study the effect of HMGB1 and its truncated form lacking the C-tail (HMGB1ΔC) on RAGE expression in NSCLC cancer cell lines with different invasive capacity: A549 with better prognosis and H1299 with negative outcome. A substantial question of interest is how HMGB1 and HMGB1ΔC influence the ratio of flRAGE to sRAGE as sRAGE is considered a decoy of RAGE ligands and the balance between the amount of soluble and full-length RAGE might be a key factor for RAGE induced dysfunction.

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′-TGCACTGGAATTCATGGGCAAAGGAGATCC-3' and reverse
5′-CAGTGCACTCGAGTTATTCATCATCATCATCTTC-3′ and for the truncated form HMGB1ΔC forward
5'-TGCACTGGAATTCATGGGC AAAGGAGATCC-3′ and reverse
5′-CTTCTTT TTCTTGCTTTTTTCAGCCTTG-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 non-small cell lung carcinoma (NSCLC) cell lines A549 and H1299 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. The synthesized recombinant proteins HMGB1 and HMGB1ΔC were purified to homogeneity as described in "Materials and methods" and used for further experiments. Both lung carcinoma cell lines were incubated for 4 h with 200 ng final concentration of protein samples when the cells had reached 70% confluency. Total protein extracts were separated on 12% PAGE, transferred to nitrocellulose membrane and visualized with specific anti-RAGE antibody. Two bands gave specific positive signal corresponding to 55 kDa MW and 38 kDa MW. Those molecular weights were assigned to the full-length RAGE (flRAGE, 55 kDa) and its soluble form sRAGE (38 kDa), respectively [14]. The signals were normalized to actin, quantified and plotted. The total amount of RAGE for lung cancer cell lines A549 and H1299 is presented in Fig.1. The results correspond to

the previous findings that in more invasive lung cancer less RAGE expression was observed [13].

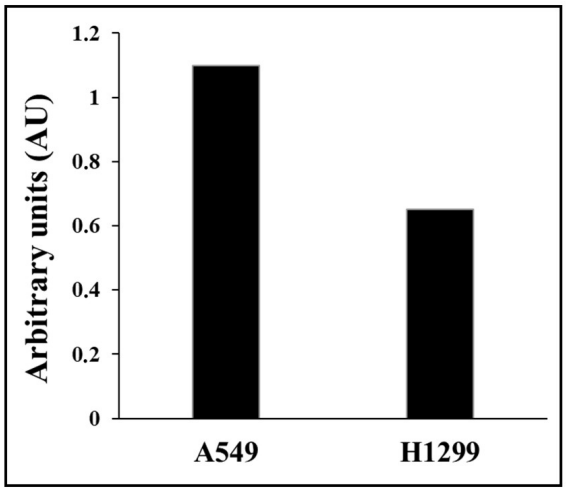

Fig. 1. Total cell extracts were run on 12% SDS PAGE, transferred on nitrocellulose membrane and visualized with specific antiRAGE antibody. The total amount of RAGE in A549 and H1299 lung cancer cell lines was quantified using Image J software and normalized to β-actin

The ratio of flRAGE to sRAGE is an important indicator for more detailed estimation of the lung cancer development as sRAGE may serve as a trap for the specific ligands and prevent cellular signalling. A crucial point also is the effect of the specific ligands which in our case are HMGB1 and HMGB1ΔC protein samples. The influence of HMGB1 and its truncated form on RAGE production in A549 cell line is presented in Fig. 2. It is important to underline that in the control cells without any ligands the total RAGE is present only as a soluble one. Upon addition of ligands two important results are observed: (i) total RAGE expression is augmented 1.5 times and 1.7 times for HMGB1 and HMGB1ΔC, respectively, and (ii), the full-length membrane variant is registered and in the presence of HMGB1ΔC its expression is comparable with the soluble one.

The effect on RAGE expression in H1299 cell line of both ligands HMGB1 and HMGB1ΔC was rather unexpected. The first difference with A549 was registered for the control cell samples (see Fig. 3). In H1299 lung cancer cells the dominant form of RAGE was the flRAGE while the soluble variant sRAGE represents approximately one third of the whole amount of the receptor. In fact, a substantial influence on total RAGE expression was detected neither for HMGB1 nor for the HMGB1ΔC upon incubation of the ligands in the H1299 cell medium. More interesting was the fact that the ratio between the membrane (flRAGE)

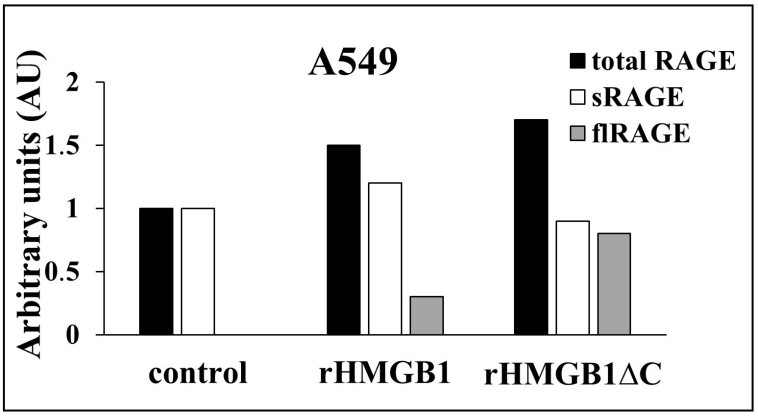

Fig. 2. The effect of specific RAGE ligands HMGB1 and HMGB1ΔC on RAGE expression in A549 lung cancer cell line

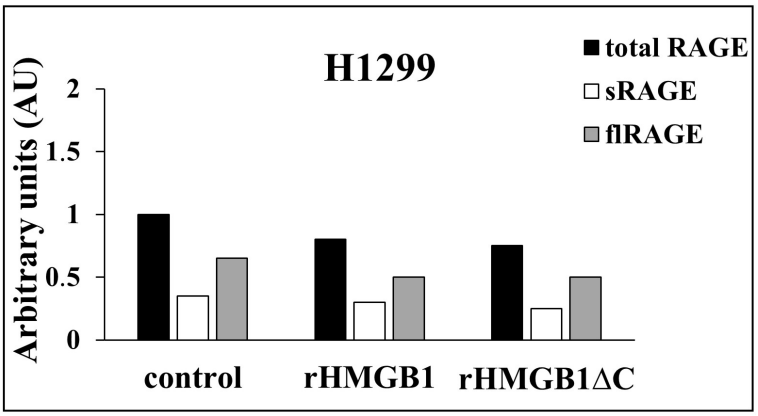

Fig. 3. The effect of specific RAGE ligands HMGB1 and HMGB1ΔC on RAGE expression in H1299 lung cancer cell line

and the soluble (sRAGE) form of the receptor was preserved in the presence or absence of specific ligands.

Discussion. The pattern of RAGE expression in lung follows opposite tendency compared to other tissues. That fact suggests the involvement of RAGE in the normal functioning of lung cells. The receptor was reported to play the role of adhesion molecule stabilizing the mature alveolar epithelial cells to their substrate by interacting electrostatically with other molecules. One explanation for the reduction of RAGE observed in lung tumourigenesis is that cell-to-cell and cell-to-substrate interaction is interrupted, which turns out to be a critical step for cancer cell progression and migration [15]. It was illustrated that knockdown of RAGE in A549 cells and human pulmonary fibroblasts resulted in increased migration [16]. The RAGE behaviour depends on the binding of specific ligands,

one of them HMGB1 protein. Importantly, RAGE ligands are not degraded or altered to prevent further signalling when they bind and signal through RAGE [6]. Therefore, as ligands accumulate, they continuously should amplify the effect on RAGE production and RAGE signalling. In our case for A549 cancer cells the presence of HMGB1 increases the total amount of the receptor and a membrane full-length RAGE appears that was absent in the control cells. The truncated HMGB1 enhances the effect on total RAGE production and the two forms of RAGE, sRAGE and flRAGE become comparable. The receptor identifies ligands based on their three-dimensional structure rather than a specific amino acid sequence [17]. The deletion of the C-tail of HMGB1 changes the protein's conformation [18], which might be a putative explanation of the observed greater effect. The results obtained for H1299 cancer cells were rather unexpected, both ligands had no effect on total RAGE expression and on the ratio of flRAGE to sRAGE. In the case of lung cancer, the higher amount of RAGE is related to better prognoses and less metastatic ability which means that the stimulation of RAGE expression by HMGB1 and HMGB1ΔC in A549 might be accepted as a positive tendency. In the case of the more aggressive lung cancer H1299 both ligands do not change the RAGE behaviour and in this way do not affect the invasive potential of the cancer cells.

What is the detailed mechanism of HMGB1/RAGE interaction for lung cancer development is still unknown. It was reported that RAGE/HMGB1 promotes the proliferation and anti-apoptosis of Lewis lung cancer cells through RAGE and toll-like receptor 4 (TLR4)-dependent signals [19]. Undoubtedly, RAGE and its isoforms play an essential role in the biology of the lung under both physiological and pathological conditions. However, it is important that future studies address the relative contribution of both RAGE and its isoforms.

REFERENCES

[1] Yonekura H., Y. Yamamoto, S. Sakurai, R. G. Petrova, M. Abedin et al. (2003) Novel splice variants of the receptor for advanced glycation end-products expressed in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury, Biochemical Journal, **370**(3), 1097–1109.

[2] Hanford L. E., J. J. Enghild, Z. Valnickova, S. V. Petersen, L. M. Schaefer et al. (2004) Purification and characterization of mouse soluble receptor for advanced glycation end products (sRAGE), Journal of Biological Chemistry, **279**(48), 50019–50024.

[3] Raucci A., S. Cugusi, A. Antonelli, S. M. Barabino, L. Monti et al. (2008) A soluble form of the receptor for advanced glycation endproducts (RAGE) is produced by proteolytic cleavage of the membrane bound form by the sheddase a disintegrin and metalloprotease 10 (ADAM10), The FASEB Journal, **22**(10), 3716–3727.

[4] Zhang L., M. Bukulin, E. Kojro, A. Roth, V. V. Metz et al. (2008) Receptor for advanced glycation end products is subjected to protein ectodomain shedding by metalloproteinases, Journal of Biological Chemistry, **283**(51), 35507–35516.
[5] Brett J., A. M. Schmidt, S. Du Yan, Y. S. Zou, E. Weidman et al. (1993) Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues, The American journal of pathology, **143**(6), 1699.
[6] Oczypok E. A., T. N. Perkins, T. D. Oury (2017) All the "RAGE" in lung disease: The receptor for advanced glycation endproducts (RAGE) is a major mediator of pulmonary inflammatory responses, Paediatric respiratory reviews, **23**, 40–49.
[7] Xie J., S. Reverdatto, A. Frolov, R. Hoffmann, D. S. Burz et al. (2008) Structural basis for pattern recognition by the receptor for advanced glycation end products (RAGE), Journal of Biological Chemistry, **283**(40), 27255–27269.
[8] Ugrinova I., E. Pasheva (2017) HMGB1 protein: a therapeutic target inside and outside the cell, Advances in protein chemistry and structural biology, **107**, 37—76, Academic Press.
[9] Scaffidi P., T. Misteli, M. E. Bianchi (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation, Nature, **418**(6894), 191–195.
[10] Li G., X. Liang, M. T. Lotze (2013) HMGB1: the central cytokine for all lymphoid cells, Frontiers in immunology, **4**, 68.
[11] Logsdon C. D., M. K. Fuentes, E. H. Huang, T. Arumugam (2007) RAGE and RAGE ligands in cancer, Current molecular medicine, **7**(8), 777–789.
[12] Franklin W. A. (2007) RAGE in lung tumors, American Journal of Respiratory and Critical Care Medicine, **175**(2), 106–107.
[13] Bartling B., H. S. Hofmann, B. Weigle, R. E. Silber, A. Simm (2005) Down-regulation of the receptor for advanced glycation end-products (RAGE) supports non-small cell lung carcinoma, Carcinogenesis, **26**(2), 293–301.
[14] Dattilo B. M., G. Fritz, E. Leclerc, C. W. Vander Kooi, C. W. Heizmann et al. (2007) The extracellular region of the receptor for advanced glycation end products is composed of two independent structural units, Biochemistry, **46**(23), 6957–6970.
[15] Marinakis E., G. Bagkos, C. Piperi, P. Roussou, E. Diamanti-Kandarakis (2014) Critical role of RAGE in lung physiology and tumorigenesis: a potential target of therapeutic intervention?, Clinical Chemistry and Laboratory Medicine (CCLM), **52**(2), 189–200.
[16] Queisser M. A., F. M. Kouri, M. Konigshoff, M. Wygrecka, U. Schubert et al. (2008) Loss of RAGE in pulmonary fibrosis: molecular relations to functional changes in pulmonary cell types, American journal of respiratory cell and molecular biology, **39**(3), 337–345.
[17] Xie J., S. Reverdatto, A. Frolov, R. Hoffmann, D. S. Burz et al. (2008) Structural basis for pattern recognition by the receptor for advanced glycation end products (RAGE), Journal of Biological Chemistry, **283**(40), 27255–27269.
[18] Elenkov I., P. Pelovsky, I. Ugrinova, M. Takahashi, E. Pasheva (2011) The DNA binding and bending activities of truncated tail-less HMGB1 protein are differentially affected by Lys-2 and Lys-81 residues and their acetylation, International journal of biological sciences, **7**(6), 691.
[19] Xu X., H. Zhu, T. Wang, Y. Sun, P. Ni et al. (2014) Exogenous High Mobility Group Box 1 Inhibits Apoptosis and Promotes the Proliferation of Lewis Cells via

RAGE/TLR 4 Dependent Signal Pathways, Scandinavian journal of immunology, **79**(6), 386–394.

Institute of Molecular Biology "Roumen Tsanev"
Bulgarian Academy of Sciences
Akad G. Bonchev St, Bl. 21
1113 Sofia, Bulgaria
e-mail: jordanabg@yahoo.com
mhristova84@abv.bg
eva@bio21.bas.bg
ugryiva@gmail.com