Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Protease-activated receptor 2 (PAR2) upregulates granulocyte colony stimulating factor (G-CSF) expression in breast cancer cells

Érika Carvalho [a], Vitor Hugo de Almeida [a], Araci M.da R. Rondon [a], Patricia A. Possik [b], João P.B. Viola [b], Robson Q. Monteiro [a, *]

[a] Institute of Medical Biochemistry Leopoldo de Meis, Federal University of Rio de Janeiro, RJ, Brazil
[b] Program of Cellular Biology, Brazilian National Cancer Institute (INCA), Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 19 August 2018
Accepted 27 August 2018
Available online xxx

Keywords:
Protease-activated receptor
Granulocyte colony stimulating factor
Breast cancer
TCGA
ENMD-1068

ABSTRACT

Protease-activated receptor 2 (PAR2) is a G-protein coupled receptor which is activated upon cleavage of its N-terminal region. PAR2 has been associated with many aspects regarding tumor progression, such as the production of pro-tumoral cytokines. Granulocyte colony-stimulating factor (G-CSF) is a cytokine essential to neutrophil production and maturation, and it is often overexpressed in tumors. In this study, we evaluated the ability of PAR2 to modulate G-CSF expression. *PAR2* and *G-CSF* were significantly more expressed in metastatic (4T1 and MDA-MB-231) as compared to non-metastatic (67NR and MCF7) breast cancer cell lines. In addition, PAR2 stimulation by a synthetic agonist peptide significantly increased *G-CSF* gene expression in the metastatic cell lines. Knockdown of PAR2 in 4T1 cells decreased G-CSF expression and secretion. In addition, treatment of 4T1 with the commercial PAR2 antagonist, ENMD-1068, significantly decreased *G-CSF* expression. cBioPortal analyses of the TCGA database showed a significant co-occurrence of *G-CSF* and *PAR2* gene overexpression in breast cancer samples. In conclusion, our data suggest that PAR2 contributes to G-CSF expression in breast cancer cells, possibly favoring tumor progression.

1. Introduction

Protease-activated receptors (PARs) are a distinct class of G protein-coupled receptors activated by proteolysis [1]. Activation of all members is initiated by N-terminal cleavage of the receptors, which exposes a unique amino acid sequence called tethered ligand. This ligand interacts with the second extracellular loop of the protein inducing conformational changes and activation of intracellular signaling pathways mainly by G proteins. PARs, more specifically PAR1 and PAR2, have been associated with several steps of cancer development and progression [2–4]. The predominant activators of PAR2 are trypsin, tryptase, binary complex tissue factor (TF)/coagulation factor VIIa (FVIIa), and ternary complex TF/FVIIa/FXa [4]. PAR2 activation promotes tumor growth, chemoresistance, migration, and angiogenesis in different cancer models [5–8]. Remarkably, overexpression of PAR2 has been associated with worse prognosis in breast cancer patients [9].

Granulocyte colony-stimulating factor (G-CSF) is a small cytokine that regulates neutrophil production in homeostasis and in response to infections. G-CSF induces proliferation of non-committed hematopoietic stem cells and granulocytic lineage due to tissue-specific gene expression of its receptor, G-CSFR [10]. Both G-CSF and G-CSFR are overexpressed in different tumor types [11–13] and several lines of evidence suggest that G-CSF supports tumor progression [14]. G-CSF is highly expressed in human breast cancer samples from the triple-negative aggressive subtype and correlates with poor overall survival [15]. Indeed, animal models demonstrate that G-CSF plays a role in tumor metastasis [16,17].

In the current study, we demonstrate that PAR2 modulates G-CSF expression in human and murine breast cancer cell lines *in vitro*. In addition, cBioPortal analyses of The Cancer Genome Atlas (TCGA) database showed significant co-occurrence of G-CSF and PAR2 overexpression in human breast cancer samples. Taken together, these results suggest that G-CSF expression might be stimulated by PAR2 signaling in tumor cells, which may be relevant for tumor progression and clinical management of patients.

* Corresponding author. IBqM/CCS/UFRJ, Avenida Carlos Chagas Filho 373 (Bloco H, segundo andar, sala 08), Cidade Universitária, Ilha do Fundão, Rio de Janeiro, 21941-599, Brazil.
E-mail address: robsonqm@bioqmed.ufrj.br (R.Q. Monteiro).

https://doi.org/10.1016/j.bbrc.2018.08.169
0006-291X/

2. Material and methods

2.1. Reagents

Human (SLIGKV-NH2) and mouse (SLIGRL-NH2) PAR2 agonist peptides (PAR2-AP) were synthesized by Biosynthesis Inc. (Lewisville, TX, USA). ENMD-1068 was purchased from Abcam (MA, USA).

2.2. Cell culture

The cells lines 4T1 (metastatic) and 67NR (non-metastatic), originated from spontaneous breast tumors in Balb/c mouse [18] were purchased from Karmanos Cancer Institute (Detroit, MI, USA). 4T1 cells were grown in RPMI 1640 medium (Gibco®; Life Technologies, MA, USA) supplemented with 10% fetal bovine serum (FBS). 67NR, as well as the human breast cancer cell lines MDA-MB-231 (metastatic) and MCF7 (non-metastatic), were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco®; Life Technologies, MA, USA) supplemented with 10% FBS. HEK293FT cell line was used for lentivirus particles packing and maintained in DMEM supplemented with 10% FBS. All supplements used were purchased from Gibco® Life Technologies (MA, USA).

2.3. Plasmid construction

The guideRNA (gRNA) for mouse *PAR2* gene were designed using the online platforms: CRISPR Design (http://crispr.mit.edu/) [19] and CRISPRscan (http://www.crisprscan.org/) [20]. The following oligonucleotides were synthesized for cloning: F: CACCGTATCACCCTTCTG GCGGCCT, R: AAACAGGCCGCCAGAAGGGTGATAC. Oligonucleotides were annealed to form double-stranded DNA in a 10 μL reaction containing: 1 μL of each primer (IDT, IA, USA), 1 μL 10× ligase buffer (New England Biolabs, MA, USA), and 7 μL water. The reaction was incubated for 30 min at 37 °C followed by 5 min at 95 °C, reducing 5 °C/min until 25 °C. Annealed oligonucleotides were ligated to BsmBI (Thermo Fisher Scientific, MA, USA) digested lentiCRISPRv2 (Addgene: #52961) by mixing: 1 μL annealed primers (diluted 1:100), 2 μL 10× T4 ligase buffer (New England Biolabs, MA, USA), 2 μL T4 DNA ligase and 15 μL of water and incubating overnight at 16 °C. Electrocompetent *Escherichia coli* XL1-Blue bacteria were transformed by electroporation. Clones were expanded and constructs were confirmed by DNA sequencing.

2.4. Production of recombinant lentiviruses and infection of target cells

HEK293FT cells (ATCC – 4×10^6 cells/plate) were plated on 10 cm diameter plates and transiently transfected by calcium phosphate precipitation on the next day. For that, 3 μg of each accessory plasmid (pRSV rev, pMDLg/pRRE and pHCMV-G) and 8 μg of the control or gRNA plasmids (empty lentiCRISPRv2 and lentiCRISPRv2-PAR2gRNA) were added to 500 μL of a 0,25 M $CaCl_2$ solution. Then, 500 μL of HEPES Buffer Saline (HBS) 2× (280 mM NaCl; 10 mM KCl; 1.5 mM Na_2HPO_4; 12 mM dextrose; 50 mM HEPES; pH7,1) was added to the transfection mixture while vortexing. Cells were incubated in media containing 25 μM chloroquine and the transfection mixture (1 mL) for 6 h. Forty-eight hours after transfection, lentivirus-containing supernatants were collected, filtered and stored at −80 °C. For lentiviral transductions, 4×10^5 of 4T1 cells were plated on 10 cm diameter plates and, on the next day, incubated with 1:2 (total: 8 ml) diluted virus supplemented with 8 μg/mL polybrene. After 24 h, cells were cultivated for 15 days in media containing 15 μg/mL puromycin to select for cells with stable integration of the expression cassette. A pooled, polyclonal cell

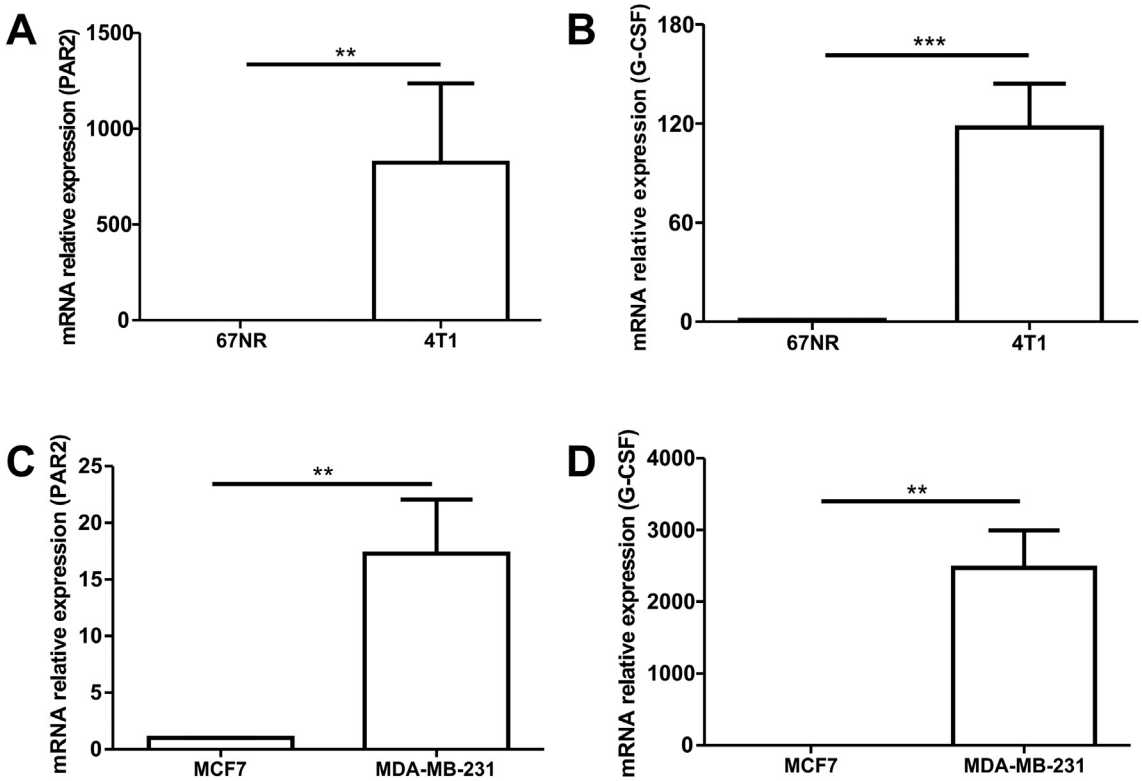

Fig. 1. PAR2 and G-CSF are upregulated in metastatic compared to non-metastatic breast cancer cell lines. Relative expression of PAR2 and G-CSF was evaluated by qPCR on 67NR and 4T1 cell lines (A, B) and MCF7 and MDA-MB-231 cell lines (C, D). Bar graphs show gene expression in metastatic cell lines relative to non-metastatic. Mouse β-actin and human TBP were used as reference genes. Values represent mean + SD of at least three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

Please cite this article in press as: É. Carvalho, et al., Protease-activated receptor 2 (PAR2) upregulates granulocyte colony stimulating factor (G-CSF) expression in breast cancer cells, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.08.169