Archives of Biochemistry and Biophysics 645 (2018) 19–25

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Integrin-mediated human glioblastoma cells adhesion, migration and invasion by native and recombinant phospholipases of *Scorpio maurus* venom glands

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ARTICLE INFO

Keywords:
Recombinant phospholipases
Scorpion venom glands
Glioblastoma
Integrins
Extracellular matrix

ABSTRACT

Integrins are a large family of cell surface receptors mediating the interaction of cells with their microenvironment and they play an important role in glioma biology. In the present work, we reported the anti-tumor effect of Sm-PLGV a phospholipase A_2 from Tunisian scorpion venom glands-as well as its recombinant forms expressed in *Escherichia coli*-through interference with integrin receptor function in malignant glioma cells U87. These phospholipases inhibited in a dose dependent manner the adhesion, migration and invasion onto fibrinogen and fibronectin without any cytotoxicity. We showed that Sm-PLGV and its recombinant constructs blocked U87 migration by reducing their velocity and directional persistence. The inhibitory effect was related to a blockage of the integrins αvβ3 and α5β1 function. Inactivation of the enzymatic activity of Sm-PLGV by chemical modification with p-bromophenacyl bromide did not affect its anti-tumor properties, suggesting the presence of 'pharmacological sites' distinct from the catalytic site in scorpion venom phospholipases A_2.

1. Introduction

Glioblastoma, also known as glioblastoma multiforme (GBM[1]), is the most aggressive cancer that begins within the brain [1]. Recent researches are focused on finding novel effective anticancer therapies, such as gene therapy, local delivery of chemotherapeutics, pore-forming toxins and targeted proteins [2]. Extensive studies have shown an alteration of the cell-adhesion activity in cancer cells. The research of new molecules able to counter this alteration can thus be considered as a promising way for the development of alternative therapies. In pharmaceutical sciences, the research of new therapeutic strategies targeting multiple cell signaling pathways, such as inhibition of proliferation, adhesion, migration, is a hopeful approach in this field. Because they play a crucial role in cancer development, integrins are particularly interesting targets [3]. Integrins are heterodimeric receptors that mediate cell attachment to the extracellular matrix (ECM[2]). They are formed by the non-covalent association of two subunits α and β [4,5]. The study of the role played by integrins has been mainly focused on αvβ3. This integrin interacts with several ECM proteins, such as vitronectin, fibrinogen and fibronectin. Besides, it cooperates with molecules endowed with different biological functions, including metalloproteinases, growth factors and their receptors [6].

Many studies have shown the effects of snake and scorpion venoms, both *in vitro* and *in vivo*, to inhibit the cancer growth, the induction of apoptosis and the suppression of cancer metastasis [7]. Recently, it has been demonstrated that venoms and toxins of some species of scorpions, especially those of the Buthidae family, have anti-proliferative effect and induce apoptosis on cancer cells *in vitro* and *in vivo* [7]. Chlorotoxin, a peptide isolated from *Leiurus quinquestriatus* venom, blocked the ion channels and bound to specific targets in the membranes of cancer cells, especially glioblastoma cells [8]. Some peptides purified from scorpion venoms, such as BmK AGAP-SYPU2 from the Chinese scorpion *Buthus martensii Karsch* and TsAP-1 and TsAP-2 from the Brazilian yellow scorpion *Tityus serrulatus*, were also able to exert a dual function with antimicrobial and antitumor activities or analgesic and antitumor activities, respectively [9,10].

Scorpion venom contains not only toxins, but also several enzymes, such as hyaluronidases, phospholipases A_2 (PLA_2[3]), L-amino acid

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[1] GBM: glioblastoma multiforme.
[2] ECM: Extracellular matrix.
[3] PLA_2: Phospholipases A_2.

https://doi.org/10.1016/j.abb.2018.03.014
Received 12 January 2018; Received in revised form 21 February 2018; Accepted 12 March 2018
Available online 13 March 2018
0003-9861/

F L I W G G T K W C G P G N V A K N E S 20
D L G P L E A D K C C R T H D H C D Y I 40
G S G E T K Y G L T N F S F F T K L N C 60
K C E E A F D Q C L T E A Y N K E E G D 80
A K T S T K D L R N F Y F D N Y R P Q C 100
Y V V T C N S K R S S R D A G C E N G V 120
A T W K K S Y K D 129

Fig. 1. Sequence of Sm-PLGV. The heterodimeric phospholipase is formed by the long chain: residue 1 to residue 107 covalently linked to the short chain: residue 113 to residue 129 (double underlined) after the release of the penta-peptide: residue 108 to residue 112 (grey box) during the maturation process. The recombinant $rPLA_2(+5)$ contains long and short chains linked by the penta-peptide while in $rPLA_2(-5)$ the penta-peptide is deleted.

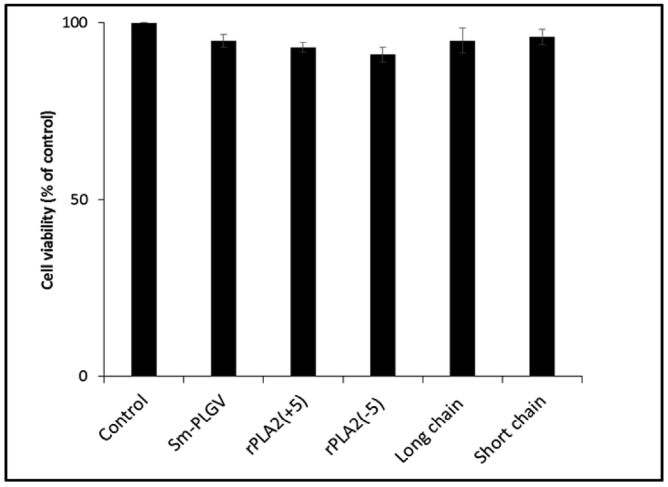

Fig. 2. Cell viability: U87 cells were cultured for 72 h with 100 µg/mL of Sm-PLGV, $rPLA_2(+5)$, $rPLA_2(-5)$, long and short chains. MTT solution was then added for 3 h and replaced by 100 µL DMSO to dissolve the precipitated formazan crystals. Finally, the absorbance was measured at 550 nm.

oxidases and proteases [7]. The PLA_2 present in scorpion venoms belong to secreted PLA_2, with a low molecular mass comprised between 14 and 19 kDa [11]. These enzymes have diverse biological and pharmacological potentials such as anti-coagulant and antibacterial activities. Moreover, they are involved in tissue destruction and inflammation [12]. The only scorpion venom PLA_2 that has an anti-tumoral effect is Hemilipin from the Iranian scorpion *Hemiscorpius lepturus*. Its anti-angiogenic activity was studied *in vitro* by evaluating its effects on apoptosis, migration and adhesion of human umbilical vein endothelial cells (HUVECs) and human pulmonary artery endothelial cells (HPAECs) and *in vivo* by the chorioallantoic membrane assay (CAM[4]) [13].

Recently, we purified and characterized a phospholipase A_2 named Sm-PLVG[5] from the venom glands of Tunisian scorpion *Scorpio maurus* [14]. This heterodimeric PLA_2, with a molecular mass 15 kDa, contains a long chain covalently linked to a short one after the release of five residues (penta-peptide) during the maturation process. Three recombinant forms were expressed in the prokaryotic system *Escherichia coli*. To investigate the importance of the different chains in the enzymatic activity, we have expressed the PLA_2 containing ($rPLA_2(+5)$[6]) or not ($rPLA_2(-5)$[7]) the penta-peptide between the long and the short chains and the long chain alone (paper in press). We showed that Sm-PLGV, $rPLA_2(+5)$ and $rPLA_2(-5)$ are able to impair *in vitro* angiogenesis by inhibiting human endothelial cells HMEC-1 adhesion, migration, invasion and tubulogenesis, as well as *in vivo* angiogenesis, by reducing the number of new capillaries and branching vessels in the CAM model (submitted paper).

In the present study, we investigated for the first time the impact of integrin inhibition by Sm-PLGV, its recombinant forms and its short chain produced by chemical synthesis on glioblastoma cells. A comparative study between the native and the recombinant enzymes is reported.

2. Material and methods

2.1. Material

p-bromophenacyl bromide (p-BPB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA) and Human fibrinogen were from Sigma Aldrich (St. QuentinFallavier, France). MEM-medium was from Lonza, Levallois-Perret, France. Fetal bovine serum (SVF), L-glutamine, penicillin and streptomycin were from Life Technologies, Paisley, UK. Bovine Type I collagen was from Upstate (Lake Placid, NY). Human fibronectin, vitronectin, laminin 1 and Poly-L-Lysine are from Chemicon (Temecula, CA). Antibodies Anti-β1 (clone Lia1/2), anti-α2 (clone Gi9), anti-αv (clone 69.6.5), anti-α5β1 (clone Sam-1) and anti-αvβ3 (clone LM609) were from Immunotech (Marseille, France).

2.2. Proteins purification

Sm-PLGV was purified from the *Scorpio maurus* venom glands as previously described [14]. Recombinant phospholipases $rPLA_2(+5)$, $rPLA_2(-5)$ and long chain which were present in inclusion bodies in *Escherichia coli*, were refolded and purified by modified method previously described [15–17]. The short chain of Sm-PLGV (17 residues) was synthetized by Bio Basic CANADA INC.

2.3. Covalent inactivation of phospholipases

Chemical modification of Sm-PLGV, $rPLA_2(+5)$ and $rPLA_2(-5)$ enzymes was performed by incubating each protein with 1 mM p-bromophenacyl bromide (p-BPB) dissolved in 0.5% dimethylsulfoxide [18]. Incubation was carried out at 4 °C for 1 h and then the excess of the reagent was removed by dialysis against 2 mM Tris-buffer pH 8. The absence of enzymatic activity was checked by pH-stat method and modified enzymes were used on different tests. In parallel, controls were run without inhibitor under the same conditions.

2.4. Cell culture and viability assay

Human glioblastoma cells U87 (ATCC) were cultured in MEM supplemented with 10% fetal bovine serum and routinely maintained at 37 °C and 5% CO_2.

Cell viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded in 96-well plates at 5000 cells/well. After 24 h, they were treated with PLA_2 forms or control vehicle and cultured for 72 h. MTT solution (500 µg/mL final concentration) was added to the culture medium 3 h before the end of

[4] CAM: chorioallantoic membrane assay.
[5] Sm-PLGV: Phospholipase A_2 purified from *Scorpio maurus* venom glands.
[6] $rPLA_2(+5)$: recombinant phospholipase A_2 containing the whole gene transcript either long and short chains linked by the penta-peptide.
[7] $rPLA_2(-5)$: recombinant phospholipase A_2 in which the penta-peptide insert is deleted.