(RESEARCH ARTICLE)

G10S an immunoglobulin peptide against melanoma

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Abstract

Peptides are the end product of transcript processing, for example allow the great viral adaptations and their transfection between species arising pandemic threats or diseases devoid of nucleic acid like prions. So, because of their high specificity, peptides can be used for different therapeutic goals. In this work we describe in vitro action of a peptide named G10S this sequence is part of a group previously selected by ExPASy Proteomics Tools Compute to find immunoglobulin molecules look like defensins of protective natural immunity which showed activity against tumors, which motivated us to investigate this activity against murine B16F10 melanoma. Thus, that criterion is capable to generate molecules against tumor, for interfering in cell adhesion, viability, migration and cycle. To conclude, the present work suggests a functional molecule against tumor.

Keywords: Peptide; Antitumor activity; Cell cycle; B16F10 melanoma; Aggressive tumor cells; Cell migration

1. Introduction

Peptides are the end product of transcript processing, allowed great viral adaptations and their transfection between species [1] arising pandemic threats. Moreover, there are also novels neurotropic viral strains capable of causing prion diseases [2,3]. Thus, if the attacker has a vaccine these viruses could be used as biological weapons against nations and political enemies [4]. So, we can understand how dynamic the protein world is.

This preliminary work aiming to collect data for future test *in vivo* so, we evaluated the *in vitro* potential for therapeutic exploitation of an immunoglobulin (Ig) peptide amidated in C-terminal named G10S, see third line in (Table 1) [5, 6,7,8].

This peptide belongs to a group of molecules encoded by immunoglobulin genes (loci lambda, kappa and heavy) resulted in the selection of four peptides with previous biological activities, denominated L12P, W12K, G10S and L18R. The amino acid sequences and characteristics of the selected peptides are shown in (Table 1) [5]. So, through these criteria we can obtain molecules similar to protective molecules of natural immunity, which by phylogenetic evolution analysis look like defensins, [9, 10]. Consequently, G10S is a genomic human immunoglobulin sequence with great potential for biological action [7, 8], what justifies the test against B16F10 murine melanoma tumor cells.

Finally, we show that peptide G10S had *in vitro* antitumor activity against B16F10, demonstrating the hypothesis that our selection criterion is capable of generating molecules that display therapeutical activity, to be tested in several antitumor experiments.

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Table 1 Characteristics of the selected peptides encoded by immunoglobulin genes

Peptide	Locus	Gene	Amino acid sequence	Hydrofhobicity[a]	pI	M.M	Net charge
L12P	Lambda	IGLJ1	LCLRNWDQGHRP	0*0+*0*0++0	8,26	1494.7	2+
W12K	Kappa	IGKJ1	WTFGQGTKVEIK	0*00*0*+00+	8,59	1393.7	+
G10S	Heavy	IGHD2-15	GYCSGGSYS	00**00**0*	5,51	983.2	0
L18R	Heavy	IGHJ2	LLVRSLGPWHPGHCLLR	0000+*0000+00+*00+	10,35	2068.1	4+

[a]0: hydrophobic, *polar, +, and −: positively charged and negatively charged residues; pI: isoelectric point; M.M.: molecular mass (Daltons).

2. Material and methods

2.1. Cell adherence assay

Viable cells were seeded (1×10^6) in a 6-well plate and treated with 1 mM peptides for 36 h. The cells were detached with trypsin (0.025%) in PBS-EDTA (0.5 mM) for five minutes at 37 °C, thus obtaining loose cells without mechanical action. Trypan blue. The number of cells in 10 µl transferred to a Neubauer chamber was counted with the aid of a manual counter. Cell adhesion was determined by the following formula: mean of four fields, multiplied by 1×10^4 as a correction factor and by 2 as a dilution factor, equivalent to the number of cells per mL. Adhesion (%) was compared to shedding of the treated cells versus the respective untreated control system. The procedure was repeated 4 times and the graphs were performed using the GraphPad Prism program.

2.2. Cell cycle analysis with propidium iodide (PI)

Control cells were incubated with dimethylsulfoxid (DMSO) and treated cells were inoculated with 1 mM of peptide G10S. Harvested tumor cells were washed twice with PBS by spinning at 300 g for 5 min and discarding the supernatant before resuspension of cells at 3×10^6 cells/mL in a cell suspension buffer (PBS + 2% fetal bovine serum, FBS; PBS + 0.1% bovine serum albumin, BSA). Cell suspensions in 500 µL buffer were aliquoted in 15 mL V-bottomed polypropylene tubes received 5 ml of cold 70% ethanol, dropwise with gently vortexing. Cells were fixed in ethanol for at least 1 h at 4 °C prior to propidium iodide (PI) staining and flow cytometric analysis. Fixed cells were washed twice in PBS by centrifugation as described above. One mL of PI staining solution at 50 µg/mL was added to the cell pellet. A final concentration 0.5 µg/mL in 50 µL of RNase A stock solution was also added to the cells and incubation was performed overnight (or at least 4 h) at 4 °C. 10^6 events of stored samples kept at 4 °C were analyzed by flow cytometry using the BD Accuri™ C6 Plus and the FlowJo software.

2.3. Cell lines and culture conditions

The murine melanoma cell line B16F10-Nex2 was originally obtained from the Ludwig Institute for Cancer Research (LICR), São Paulo branch. The cell line grew in RPMI-1640 (Gibco, Grand Island, NY) medium supplemented with 10 mM of 2-(4-(2-hydroxyethyl) piperazin-1-yl) ethanesulfonic acid (HEPES; Sigma-Aldrich, St. Louis, MO), 24 mM sodium bicarbonate, 40 mg/L gentamicin (Hipolabor, Minas Gerais, Brazil), pH 7.2, and 10% fetal bovine serum (Gibco, Grand Island, NY). Cells were cultured at 37 °C and 5% CO_2 and 95% humidity in the atmosphere.

2.4. "MTT" formazan cell viability assay

In a 96-well plate, 10^3 B16F10-Nex2 cells were incubated with a serial dilution of G10S, from 1 mM to 0.125 mM, and DMSO+PBS as negative controls for 36 h. A 5 mg/mL stock solution of "MTT" 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan, Thiazolyl blue formazan) was prepared in sterile H_2O and 10 µL was added to each well containing 100 µL culture medium. After 4 h, the MTT containing-medium was removed from each well with a pipette and 100 µL of 10% SDS in H_2O was added to solubilize the formazan crystal. The solutions were analyzed in a SpectraMax M2e (Molecular Devices) measuring the absorbance at 550 nm with a 690 nm filter with values plotted and analyzed on GraphPad Prism software.

2.5. Selection and synthesis of peptide encoded by immunoglobulin gene

The peptide sequences used in the present work is GYCSGGSCYS-NH2, amidated in C-terminal, called peptide G10S. The peptide were obtained by the following criteria: presence of positively charged residues, net charge, isoelectric point,

and alternation of hydrophobic/hydrophilic residues in the sequence, by using ExPASy Proteomics Tools Compute pI/MW and ProtParam. This sequence was obtained from human immunoglobulin locus heavy diversity 2 (IGHD2-15). Hydrophobicity (00**00**0*). Isoelectric point (pI) 5.51. Molar mass (M.M) 983.2. Net charge 0. The research exploiting the Gene database of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov). Selected peptides were synthesised by solid phase peptide synthesis method using a multiple peptide synthesiser (SyroII, MultiSynTech GmbH), at CRIBI Biotechnology Center (University of Padua, Italy). The purity of peptides, evaluated by analytical reverse phase HPLC, was in the 80–90% range. The peptides were solubilised in dimethylsulfoxide (DMSO) at a concentration of 20 mg/ml and subsequently diluted in sterile distilled water for experimental use. For all experiments, controls (in the absence of peptides) contained dimethylsulfoxide at the proper vehicle concentration.

2.6. Wound-healing assay for cell migration

B16F10-Nex2 cells (3×10^5) were seeded in 12-well plates and allowed to adhere and replicate to 70-80% confluence. Peptides were added at 1 mM on adherent cells and further incubated for 12 h before scratching the cell monolayer with a sterile 1,000-μL-micropipette tip on each well. Cellular debris were removed after scratching and smoothing the edge of the scratch was achieved by washing the cells once with 1 ml of the culture medium and then replacing with 5 mL culture medium containing the peptides for the *in vitro* wound healing assay. Cells incubated with DMSO at a concentration of 20 mg/ml were used as control.

For image acquisition, the distances were calibrated with a micrometer blade; tip marks bordering the scratch were made on the outer bottom of the dish. Finally, the dish is examined in a phase-contrast microscope, leaving the reference mark outside the capture image field. Images were taken at 12 h regular intervals up to 36 h and the distance of cell migration was measured using ImageJ software. By comparing the images from time 0 with those at other time points, we quantified the distance of each scratch closure determined by the cell front and measured as indicated above.

3. Results and discussion

At first, was used Trypan blue exclusion staining to analyze and check if the maximal of 1 mM doses used in the experiments, avoid any unspecific death and being safer to answer the experimental questions. Since G10S peptide presented a toxic effect at concentrations superior of 1.5 mM (data no shown),
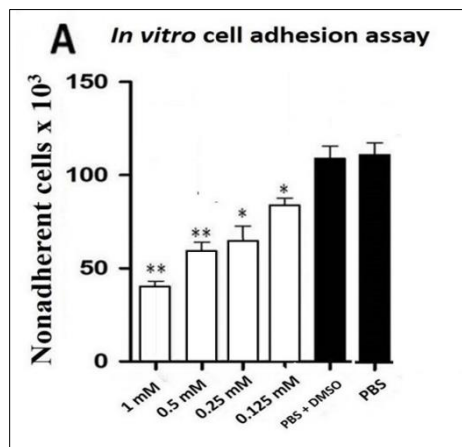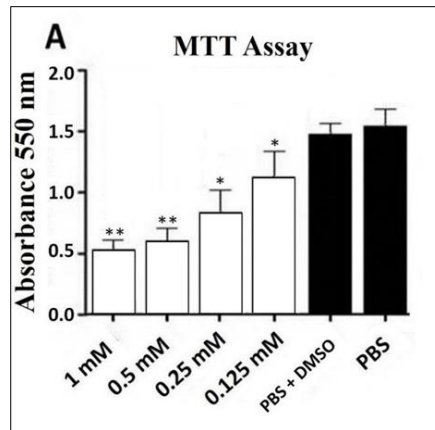

(**A**) The number of free cells was higher in controls PBS+ DMSO and PBD (black bars) compared to B16F10 cells cultured in the presence of G10S with serial dilutions of 1 mM, 0.5 mM, 0.25 mM and 0.125 mM for 36 hours (white bars). The graphs were made using the GraphPad Prism software and using Bonferroni's Multiple Comparison and T-test as a statistical analysis. (* = $p < 0.05$) and (** $p < 0.01$)

Figure 1 *In vitro* cell adherence assay

To verify the direct effect of G10S peptide on in vitro cell adhesion, we have used a cell adherence assay that showed an increased adhesion rate in melanoma cells as compared to control (Figure. 1). So, the treatment with G10S probably increased the expression levels of adhesion protein E-cadherin and decrease in N-cadherin in cells (Figure. 1), a sign of decreased tumor cell aggressiveness, since the increased expression of E-cadherins keeps the tumor cells fixed to the source organ and decrease metastasis but N-cadherin allows fixation in other organs contributing to increased metastases [11,12]. So, to answer the question how G10S effects cell adhesion, and if the peptide could reverse a cadherin adhesion aggressive phenotype, based on this result, these data need to be confirmed through Western blotting (WB).

The MTT assay showed that G10S treatments (0,125 mM, 0,25 mM, 0.5 mM, and 1 mM) decreased melanoma cell viability compared to controls after 36h (Figure. 1). This result indicates that the G10S acts on cell viability, thus being possible to also test cell proliferation in future studies.

(**A**) Absorbance reading at a wavelength of 550 nanometers (nm) of B16F10 cells cultured in the presence of G10S with serial dilutions of 1 mM, 0.5 mM, 0.25 mM and 0.125 mM for 36 hours. The MTT viability assay showed a statistically significant difference between G10S treated cells at 1 mM, 0.5 mM and 0.25 mM versus controls PBS and PBS + DMSO. The graphs were made using the GraphPad Prism software and using Bonferroni's Multiple Comparison and T-test as a statistical analysis. (* = $p < 0.05$) and (** $p < 0.01$).

**Figure 2** Viability of B16F10 cells treated with G10S

The migration assay was used to answer the question if G10S peptide can inhibit tumor cell migration. Thus, the presence of G10S in middle at 1 mM significantly reduced cell migration capacity. After 36 h of incubation, the G10S-treated cells wound was still unfilled, as seen on (Figure. 3A). In contrast, after 36 h, control PBS –treated cells continued to completely fill the wound gap (Figure. 3 B), was also verified that control PBS+DMSO behaved like the PBS–treated cells, with normal cell migration to fill the wound (Figure. 3 C). This way, we detect a direct effect of the peptide G10S on cell migration, aiming for a future clonogenic assay to observe the toxic effect of the peptide.
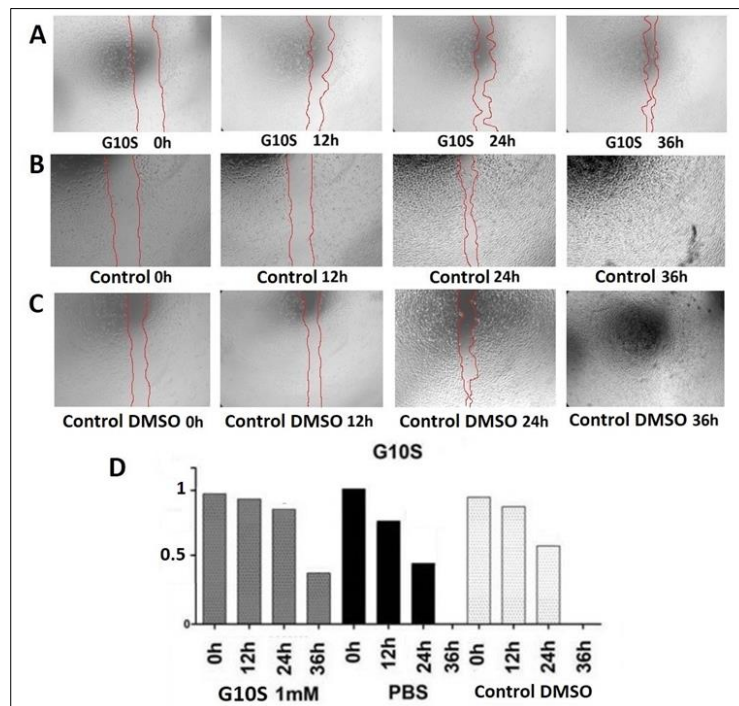
The G10S peptide was added at 1 mM concentration and images were taken at regular intervals of 12 h; the graphics represents the area between cells, black PBS control, gray treated G10S and light gray PBS + DMSO control. The area of cell migration was measured in μm using the ImageJ software. (**A**) cells that received G10S the gap was still open after 36 h (**B**) Using PBS as control, the wound was completely healed after 36 h; (**C**) Using PBS + DMSO, the wound was completely healed after 36 h

Figure 3 Wound-healing assay after G10S treatment

As a last experiment we used flow cytometry to analyze the cell cycle of melanoma cells stained with propidium iodide (Figure. 3 A-C). Thereby there are two cellular checkpoints to move cells from G1 phase to S phase [13]. The first checkpoint involves cyclin dependent kinase 2 (CDK2) interaction with cyclin E, forming the cyclin E-CDK2 complex that sequesters p27 Kip and allowing the cell to enter in S phase. The second checkpoint evolves Cyclin D1, a complex working as a regulatory subunit of CDK4 and of CDK6, whose activity is required for cell cycle G1/S transition and by interaction with proteins p27 Kip, allowing cell entry into the S phase [14]. In treatment in vitro we suggesting that the G10S could be competing in the interaction and degradation pathway involving P27kip [13,14] Thus, a raise in the amount of p27kip suggest an interference in the degradation of cyclin D caused by the G10S because the cyclin E/CDK2 and CDK4 complex phosphorylates p27Kip1, which promotes degradation of cyclin D1 as well as cyclin E with progression to S phase and cyclin A degradation with progression to G2 phase [14] consequently causing a delay in the cell cycle progression as well as observed in (Figure. 3 B-C). Lastly, the effect on cell cycle could be explained by the interaction of G10S with these cell cycle molecules, thereby the tumor cells are unable to precisely make the check point between the G1 and S phases and as a consequence of this, there was an expansion of the S phase and a shrinkage of G2 /M phases observed in (Figure. 3 B-C).; so, we intend to clarify the data with new experiments by Western blotting of the cell cycle signaling paths.
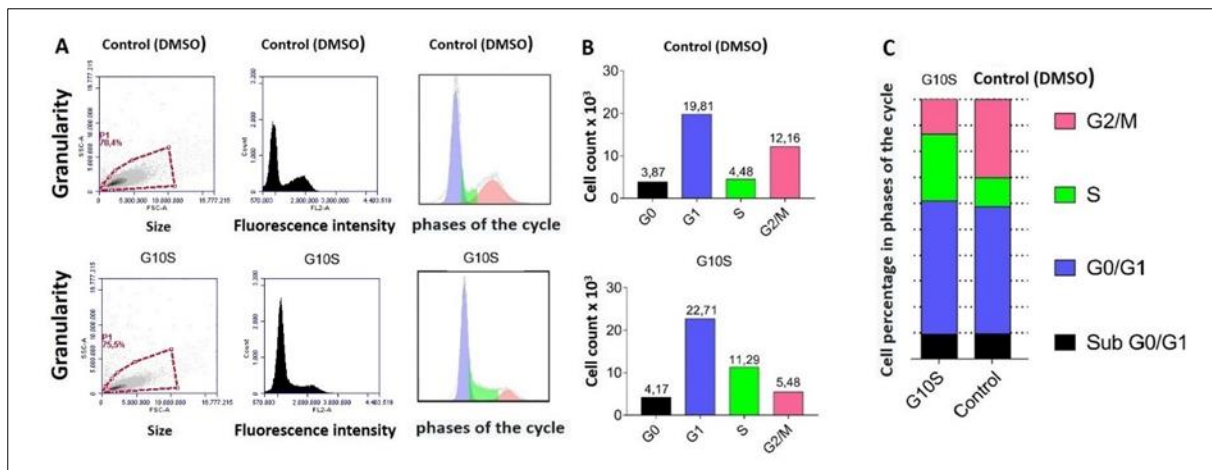
10^6 B16F10 cells are treated with G10S and analyzed by flow cytometer after incubation with propidium iodide (PI) solution. (**A**) PI staining shows different intensities in the population of G10S treated cells with difference in cell cycle pattern in comparison to PBS + DMSO control. (**B**) Number of cells in each phase of the cycle showed G10S treated and control PBS + DMSO in G1, S and G2 cell cycle phases. (**C**) Percentage of each phase of the cycle in G1, S and G2 phases. The graphs and analyzes were made using FlowJo and GraphPad software

Figure 4 Cell cycle modulation with G10S treatment

4. Conclusion

The G10S acts on cell adherence, viability and cycle inhibiting tumor cell migration and disturbing normal cycle. The increase in S phase, with decreasing G2M phase, are typically caused by the delay in the cell cycle, which can explain the observed data in MTT and cell migration of G10S-treated melanoma cells.

To finish, we show that G10S had an *in vitro* antitumor activity against B16F10, demonstrating an hypothesis that our selection by ExPASy Proteomics Tools Compute criterion is capable to find immunoglobulin molecules look like defensins of protective natural immunity, to obtain molecules that display biological activity, against melanoma *in vitro* thus permit tests in many other types of *in vivo* cancer models.

Compliance with ethical standards

Statement of informed consent

We authorize the full disclosure of the manuscript text and data.

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