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Fasting boosts sensitivity of human skin melanoma to cisplatin-induced cell death

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ABSTRACT

Melanoma is one of leading cause of tumor death worldwide. Anti-cancer strategy includes combination of different chemo-therapeutic agents as well as radiation; however these treatments have limited efficacy and induce significant toxic effects on healthy cells. One of most promising novel therapeutic approach to cancer therapy is the combination of anti-cancer drugs with calorie restriction. Here we investigated the effect Cisplatin (CDDP), one of the most potent chemotherapeutic agent used to treat tumors, in association with fasting in wild type and mutated $BRAF^{V600E}$ melanoma cell lines. Here we show that nutrient deprivation can consistently enhance the sensitivity of tumor cells to cell death induction by CDDP, also of those malignancies particularly resistant to any treatment, such as oncogenic BRAF melanomas. Mechanistic studies revealed that the combined therapy induced cell death is characterized by ROS accumulation and ATF4 in the absence of ER-stress. In addition, we show that autophagy is not involved in the enhanced sensitivity of melanoma cells to combined CDDP/EBSS-induced apoptosis. While, the exposure to 2-DG further enhanced the apoptotic rate observed in SK Mel 28 cells upon treatment with both CDDP and EBSS.

1. Introduction

The incidence of melanoma has been increasing significantly over the years. Although is the less frequent type of skin cancer, it is the most lethal due to the high incidence of metastasis and lack of effective treatments [1,2]. Although UV-radiation exposure represents the main cause of melanomagenesis it is now evident the association also with several gene mutations [3,4]. Among these, the RAS/RAF/MEK pathway seems to be the most affected, with $BRAF^{V600E}$ present in approximately 70% of all melanomas [5,6]. The mutation leads to constitutively active enzyme which determines an uncontrolled cell proliferation, inactivation of cell death pathways, ER stress and autophagy dysregulation, and resistance to any treatment [7]. This mutation was also correlated with alterations in the metabolic profile of melanoma cancer cells, favoring the glycolytic metabolism and reducing the rate of mitochondrial catabolic pathways [8]. This is in accordance with the "Warburg effect", one of the hallmarks of cancer cells that have an alteration of metabolic pathways compared to normal cells. One of the most important change is the up-regulation of glycolysis as a primer source of energy and building blocks even in the presence of abundant oxygen [9,10]. However, the metabolic profile is high heterogeneous in melanoma cells, even between those harboring a $BRAF^{V600E}$ mutation, which render difficult the use of a metabolism targeted therapy [11].

It has been recently demonstrated that caloric intake modulation can influence both the development and treatment outcome of many cancers, in part by reprogramming the metabolism [12–14]. One of these protocols consists in severe nutrients restriction cycles (fasting) combined with chemotherapeutic treatment, which increases the susceptibility of cancer cells to cell death [12]. Dietary

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interventions also up-regulates the AMPK signalling, an important pathway with different targets for cancer treatment [15]. This pathway is also involved in autophagy regulation [16], a possible mechanism involved in the action of fasting since during the stress induced by nutritional deprivation autophagy is up-regulated as a survival mechanism [17]. Though, it is known that aggressive tumors that harbor Ras mutations are energetically compromised under starvation conditions since this mutation limits a higher induction of the already increased basal autophagy. In this way there is not enough energy supply, leading tumor cells to cell death [18,19].

Cisplatin (CDDP) is one of the most potent chemotherapeutic agent used to treat a wide range of malignancy as lung cancers, head and neck tumors, ovarian carcinoma, among others [20]. Before the development of targeted therapies, cisplatin was one of the first choice to treat melanoma, however with a poor response rate [1]. CDDP resistance may occur at several levels as the cellular extrusion of the drug [21], mechanism of DNA repair activation [22] and autophagy induction [23]. Nevertheless, identifying new uses for existing drugs might be an effective therapeutic approach to overcome drug resistance in diverse malignancy and sustain patient's survival [29]. Therefore, we aimed to evaluate the opportunity of using fasting cycles combined with cisplatin treatment as a novel valuable clinical approach to treat human skin melanoma independently of its mutational status.

2. Materials and methods

2.1. Materials

Cisplatin (Sigma Aldrich), Bafilomycin A1 (Sigma Aldrich), Z-VAD-fmk (Santa Cruz Biotechnology), Necrostatin-1 (Sigma Aldrich), *N*-acetyl-cysteine (Sigma Aldrich), 2-deoxy-glucose (Sigma Aldrich), EBSS (Sigma Aldrich).

2.2. Cell culture

CHL-1 and SK Mel 28 metastatic melanoma cells were cultured in DMEM high glucose (Sigma-Aldrich) supplemented with 10% FBS (Life Technologies), 2 mM L-glutamine, and 1% penicillin/streptomycin solution, at 37 °C under 5% CO_2.

2.3. Apoptosis evaluation

Briefly, 1,5 × 10^5 CHL-1 or SK Mel 28 cells were treatment as indicated, for 24 h. Then, cells were fixed with methanol-acetone, pelleted and resuspended in RNAse (50 μg/ml in PBS) and incubated at 37 °C for 15 min followed by propidium iodide (25 μg/ml in PBS) staining. The cell cycle distribution was evaluated by flow cytometry. 10.000 events were acquired using a FACS Calibur cytometer (Becton-Dickinson, Mountain View,CA, USA) and the sub-G1 phase analyzed using FlowJo software [24].

2.4. ROS and $\Delta\Psi m$ assessment

Briefly, 1,5 × 10^5 CHL-1 or SK Mel 28 cells were treatment as indicated and cells harvested at indicate time points. Then, cells were pelleted and resuspended in H_2-DCFDA (10 μM in PBS) or in TMRM (50 nM in PBS), to detect ROS or to evaluate the $\Delta\Psi m$, respectively. Cells were incubated at 37 °C for 15 min in the dark, and 10.000 events were acquired by using a FACS Calibur cytometer (Becton-Dickinson, Mountain View,CA, USA). Data analysis was performed using FlowJo software.

2.5. Real time PCR analysis

Total RNA was extracted by using Trizol reagent (Invitrogen) as recommended by the supplier. cDNA synthesis was performed using a reverse transcription kit (Promega) according to the manufacturer's recommendations. Quantitative PCR reactions were performed by using a Rotor-Gene 6000 (Corbett Research Ltd) thermocycler. Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) was used to produce fluorescently labeled PCR products. Primer sets for all amplicons were designed using Primer-Express 1.0 software (Roche).

ATF4 forward: 5′-GTGGCCAAGCACTTCAAACC-3′
ATF4 reverse: 5′-CCCGGAGAAGGCATCCTC-3′
ATF6 forward: 5′-TTTGCTGTCTCAGCCTACTGTGGT-3′
ATF6 reverse: 5′-TCCATTCACTGGGCTATTCGCTGA-3′
Xbp-I forward: 5′-GAATGAAGTGAGGCCAGTG-3′
Xbp-I reverse: 5′-GAGTCAATACCGCCAGAATC-3′
L34 forward: 5′-GTCCCGAACCCCTGGTAATAGA-3′
L34 reverse: 5′-GGCCCTGCTGACATGTTTCTT-3′

L34 mRNA level was used as an internal control and results were expressed as previously described [24].

2.6. Western blotting analysis

Cells were lysed in Cell Lytic buffer (Sigma-Aldrich) supplemented with protease and phosphatase inhibitors (protease inhibitor cocktail plus 5 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1 mMsodium molybdate, 50 mM 2-chloroacetamide, 2 mM 1,10-phenanthroline monohydrate, and 0,5 mM PMSF; Sigma-Aldrich). Lysates were incubated at 4 °C for 30 min in ice. After centrifugation at 4 °C for 10 min at 13,000 rpm to remove insoluble debris, protein concentration was determined using a Bradford assay (Biorad). 20 μg of total proteins were resolved by using NuPAGEBis-Tris gels (Life Technologies) and electroblotted onto nitrocellulose (Protran, Schleicher&Schuell) or PVDF (Millipore) membranes. Blots were incubated with primary antibodies resuspended in 5% non-fat dry milk in PBS plus 0.1% Tween-20 overnight at 4 °C. Detection was achieved using a horseradish peroxidase-conjugate secondary antibody (1:5000 in 5% non-fat dry milk in PBS plus 0.1% Tween-20, Jackson Immuno Research Laboratories), visualised with ECL (GE Healthcare) and images were recorded by using a Chemidoc apparatus (BioRad). Primary antibodies were: anti-PARP cleaved (1:1000, Cell Signalling), anti-p62 (1:2000 MBL), anti-LC3B (1:1000, Cell Signalling), anti-Ambra1 (1:1000, Cell Signalling), anti-Beclin-1 (1: 500, Santa Cruz Biotechnology); anti-Atg5 (1:500, Santa Cruz Biotechnology); anti-Actin (1:5000, Santa Cruz Biotechnology) and anti-GAPDH (1:20000, Calbiochem).

2.7. Statistics

All values are represented as the mean ± SD. Significance was evaluated by ANOVA one-way followed by Tukey test for multiple comparisons among control and treatments. ANOVA two-way followed by Bonferroni post-test was used for group analysis. Differences were considered significant with $p \leq 0.05$. At least three independent experiments were conducted to warrant that the results were representative.

3. Results and discussion

3.1. Starvation enhances melanoma cells sensitivities to cisplatin-induced cell death

Previous results show severe nutrient restriction (starvation)

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