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Nitric oxide donor augments antineoplastic effects of arginine deprivation in human melanoma cells

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A B S T R A C T

Anticancer therapy based on recombinant arginine-degrading enzymes has been proposed for the treatment of several types of malignant cells deficient in arginine biosynthesis. One of the predicted side effects of such therapy is restricted bioavailability of nitric oxide as arginine catabolic product. Prolonged NO limitation may lead to unwanted disturbances in NO-dependent vasodilation, cardiovascular and immune systems. This problem can be overcome by co-supplementation with exogenous NO donor. However, NO may potentially counteract anticancer effects of therapy based on arginine deprivation. In this study, we evaluate for the first time the effects of an exogenous NO donor, sodium nitroprusside, on viability and metastatic properties of two human melanoma cell lines SK-MEL-28 and WM793 under arginine-deprived conditions. It was revealed that NO did not rescue melanoma cells from specific effects evoked by arginine deprivation, namely decreased viability and induction of apoptosis, dramatically reduced motility, invasiveness and clonogenic potential. Moreover, sodium nitroprusside co-treatment augmented several of these antineoplastic effects. We report that a combination of NO-donor and arginine deprivation strongly and specifically impaired metastatic behavior of melanoma cells. Thus, sodium nitroprusside can be considered as an adjuvant for the more efficient treatment of malignant melanoma and possibly other tumors with arginine-degrading enzymes.

1. Introduction

Cutaneous malignant melanomas, one of the most aggressive forms of cancer, are often refractory to classic chemotherapeutic drugs, immunotherapy, targeted therapy or their combination [1,2]. The majority of malignant melanomas are also known to exhibit metabolic defects that lead to their dependency on extracellular supply of arginine [3,4]. Arginine auxotrophy in melanoma cells arises from down-regulated expression of argininosuccinate synthetase (ASS), an enzyme of arginine anabolism and urea cycle [5,6]. It was recently shown that the defect of ASS expression evokes increased proliferative activity of melanoma cells due to redirection of ASS substrate aspartate for nucleotide synthesis [7]. Therefore, systemic depletion of free circulating L-arginine with arginine-degrading enzymes, e.g., bacterial arginine deiminase (ADI) or recombinant human arginase I (rhARG1), has been proposed as metabolic therapy against ASS-negative melanomas. Currently, these arginine-degrading enzymes are being evaluated in phase I/II clinical trials, in particular against metastatic melanoma [6,8,9]. Also, different combinations of these enzymes and conventional chemotherapeutic drugs for melanoma and other tumors treatment are being actively evaluated *in vitro* [10–12].

Although systemic depletion of arginine was demonstrated to be tolerated by human organism, it may nevertheless lead to a concomitant deficit in arginine metabolic products. One of arginine catabolites, NO, is a multifunctional signaling molecule, implicated in plethora of physiological processes, such as neurotransmission, vasodilation, platelet aggregation, smooth muscle relaxation, immunological response and others [13,14]. Thus, long-term arginine depletion in humans may potentially lead, due to NO limitation, to vasoconstriction, thrombosis and functional impairment of the immune system.

Up to date, the possible disturbances of NO homeostasis evoked by enzymatic arginine deprivation have not been sufficiently studied. It was reported that ADI treatment inhibited cytokine-induced NO production in endothelial cells [15], and that ADI and rhARG1

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suppressed NO synthesis in murine macrophages [16]. Animal studies with arginine-degrading enzymes revealed that NO homeostasis was indeed altered. A drop in NO plasma levels in a melanoma xenograft mouse model as well as inhibition of NO synthesis in mice treated with TNF-α and endotoxin were observed under ADI administration [17,18].

Although there was no increased blood pressure or heart rate upon ADI treatment of patients with metastatic melanoma [8], the development of portal vein thrombosis was observed under the same treatment in some patients with hepatocellular carcinoma [19]. Prolonged NO limitation is accompanied by a higher risk of developing venous and arterial thromboembolic damages in patients with metastasis cancer, and its decrease may cause acute myocardial infarction [20]. Of note, it is unclear at the moment how exactly arginine deprivation *via* recombinant arginase affects blood and intracellular levels of its precursor citrulline, which in tightly coupled reactions mediated by ASS and argininosuccinate lyase (ASL) can be converted in epithelial cells to arginine and ultimately to NO by NO-synthases.

One may assume that NO bioavailability can be restored *in vivo* by co-supplementation with an exogenous NO donor, given that the latter does not improve tumor cell survival or counteracts other effects elicited by arginine starvation.

It should be taken into account that the role of NO in tumor physiology and progression is very controversial [21]. The effects of different NO donors as sensitizing agents in combination with chemotheurapetic drugs on apoptosis progression in cancer cells *in vitro* and *in vivo* and possible mechanisms involved have been summarized earlier [22]. The NO role in regulation of such features of malignant cells as cell motility, adhesiveness and invasiveness is also complex and not fully understood [23–25].

In this study, we addressed the question as of how exogenous NO donor, sodium nitroprusside (SNP), affects viability, proliferative potential and metastatic properties of cultured melanoma cells under arginine deprivation. Metastatic, amelanotic human melanoma SK-MEL-28 and vertical growth phase primary melanoma WM793 were chosen as experimental cell models for this study. Both cell lines have mutated ^{V600E}B-RAF and wild type N-RAS [26,27].

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), dialysed FBS, collagen type I, gentamycin, sodium nitroprusside (SNP), trypan blue, crystal violet, (4,5-dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (USA). Antibodies against the following antigens were used: p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-p38 MAPK (Thr180/Tyr182), p38 MAPK, p-S6 Ribosomal Protein, S6 Ribosomal Protein, 4E-BP1, c-PARP1, PARP from Cell Signaling, Danvers, USA; NOS2 (sc-651) from Santa Cruz Biotechnology, Heidelberg, Germany; ASS1 (AM06726PU-N) from Acris Antibodies, Herford, Germany; OTC (HPA000243) from Atlas Antibodies AB, Stockholm, Sweden; β-actin (A5441) from Sigma-Aldrich, St. Louis, USA. Secondary goat anti-mouse and anti-rabbit antibodies, enhanced chemiluminescence system (ECL) and PVDF membrane were purchased from Millipore Corporation (USA). Complete protease inhibitor cocktail was purchased from Roche Applied Science (Germany).

2.2. Cell culture and treatments

Two human melanoma cell lines – SK-MEL-28 (derived from a malignant melanoma) and WM793 (derived from a primary melanoma) were used in this study. SK-MEL-28 was obtained from Institute of Cell Biology and Immunology, University of Stuttgart (Stuttgart, Germany). WM793 cell line was kindly provided by Dr Anna Lityńska (Jagiellonian University, Krakow, Poland). Cell cultures were routinely tested for mycoplasma contamination.

Melanoma cell lines were cultured in DMEM containing 1 g/L glucose, 1% sodium pyruvate, 1% glutamine and 3.7% $NaHCO_3$, supplemented with 10% heat-inactivated FBS, and 50 µg/ml gentamycin in a humidified 5% CO_2 incubator at 37 °C. Cell were subcultivated every 2–3 days by trypsinization, split in a 1:3 ratio and were used in the exponential growth phase in all experiments. In all experiments, the medium was formulated such that it could either contain 0.4 mmol/l arginine (complete arginine-rich medium, CM) or be arginine-free (AFM). Experimental media were supplemented with 5% dialysed FBS.

For adhesion, aggregation, wound healing, and invasiveness assays cells were treated with or without SNP (0.1 mM) during 72 h or AFM, trypsinized and cell suspensions in a different cell density were seeded in appropriate plates.

2.3. Cell viability assay

The dynamic of cells growth was determined by trypan blue exclusion test and cell viability by MTT assay were determined as described earlier [28]. Growth inhibition was expressed as percentage of the corresponding control cells. All experiments were conducted using triplicate determinations per plate and each assay was repeated at least three times. The concentration of SNP required to kill 50% of the cells in a culture was defined as the inhibitory concentration (IC50).

2.4. Western blot analysis

Western blot analyses were conducted using whole cell protein extracts from cell cultures according to the established and described earlier protocol [29].

2.5. Cell clonogenic survival assay

Clonogenic assay was used to determine cell ability to form colonies after 72 h cell exposure to arginine-free medium with/or without 0.1 mM SNP. 1000 cells were plated in 35 mm culture dishes. After 72 h above mentioned treatment, medium was replaced into arginine-rich medium and cells were grown for 15 days. When the cell colonies grew to approximately 50 cells and had more then 60 µm in diameter the media were removed, plates were washed with PBS, fixed by 70% ethanol for 10 min and stained with 0.5% crystal violet for 10 min at room temperature. Then, plates were washed with distilled water to remove excess crystal violet, dried, and the violet stained colonies were counted. The experiments were performed in triplicate and repeated three times.

2.6. Slow aggregation assay

Cells after indicated treatments (see “Cell culture and treatments”) were trypsinized and 250,000 cells were plated into 60-mm agarose-coated cell culture dishes and incubated for 24 h. The aggregates were subsequently examined under an inverted phase-contrast microscope, photographed. The cell viability within aggregates was evaluated by trypan blue exclusion. The agarose-coated dishes were prepared by coating the plates with 2 ml of 1.5% agarose solution in DMEM medium without serum. All experiments were repeated three times in triplicates.

2.7. Cell adhesion assay

Cell suspensions of $4{\times}10^5$ SK-MEL-28 cells and $5{\times}10^5$ WM793 cells after each treatment were seeded into 96-well plates coated collagen type I in concentration 50 µg/ml. The working solution of type I collagen (50 µg/ml) was prepared by diluting with PBS contain-

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