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Original Article

Everolimus selectively targets vemurafenib resistant BRAF^{V600E} melanoma cells adapted to low pH

Jessica Ruzzolini [a, 1], Silvia Peppicelli [a, 1], Elena Andreucci [a], Francesca Bianchini [a], Francesca Margheri [a], Anna Laurenzana [a], Gabriella Fibbi [a], Nicola Pimpinelli [b], Lido Calorini [a, c, *]

[a] Department of Experimental and Clinical Biomedical Sciences "Mario Serio", Section of Experimental Pathology and Oncology, University of Florence, Italy
[b] Department of Surgery and Translational Medicine (DCMT), University of Florence, Italy
[c] Istituto Toscano Tumori, Center of Excellence for the Study at Molecular and Clinical Level of Chronic, Degenerative and Neoplastic Diseases to Develop Novel Therapies (DENOTHE), Italy

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ABSTRACT

Vemurafenib, a BRAF inhibitor, elicits in ~80% of BRAF^{V600E}-mutant melanoma patients a transient anti-tumor response which precedes the emergence of resistance. We tested whether an acidic tumor microenvironment may favor a BRAF inhibitor resistance. A375M6 BRAF^{V600E} melanoma cells, either exposed for a short period or chronically adapted to an acidic medium, showed traits compatible with an epithelial-mesenchymal transition, reduced proliferation and high resistance to apoptosis. Both types of acidic cells treated with vemurafenib did not change their proliferation, distribution in cell cycle and level of *p*-AKT, in contrast to cells grown at standard pH, which showed reduced proliferation, cell cycle arrest and ERK/AKT inhibition. Even after treatment with trametinib (MEK inhibitor) acidic cell features did not change. Then, since both types of acidic cells exhibited high p-p70S6K, i.e. active mTOR signaling, we tested everolimus, an mTOR inhibitor, which was efficient in inducing apoptosis in acidic cells without affecting melanoma cells grown at standard pH.

Our results indicate that an acidic microenvironment may cooperate in inducing a BRAF inhibitor resistance in melanoma cells and a combined therapy with everolimus could be used to overcome that resistance.

Introduction

Melanoma is a very aggressive skin cancer and its incidence has dramatically increased during the last decades. More than ~50% of melanoma is characterized by point mutations of BRAF (V-raf murine sarcoma viral oncogene homolog B1) that is a regulator of the MAPK pathway and the most common mutation displays a valine to glutamic acid substitution (V600E) causing constitutive kinase activation [1]. The discovery of V600E point mutation encouraged the generation of compounds specifically targeting this hyperactive mutated kinase. One of these compounds, vemurafenib (also known as PLX4032) an orally ATP-competitive small-molecule inhibitor, received FDA approval for the treatment of late-stage melanoma on August 17, 2011.

Vemurafenib selectively inhibits the ATP-binding site of BRAF^{V600E} kinase resulting in an inhibition of over-activated MAPK signaling with reduction of tumor cell proliferation. Despite the initial benefits due to the treatment with vemurafenib, almost all patients develop drug resistance after weeks to months of therapy [2–5]. Documented mechanisms of BRAF inhibitor resistance are under study and up-to-now include MAPK signaling reactivation through expression of alternative splicing forms of BRAF^{V600E}, amplification of BRAF^{V600E}, mutations in NRAS or MEK (MAP2K1), or loss of NF1 [6–10]. Another mechanism of resistance includes

Abbreviations: MAPK, mitogen-activated protein kinase; RTK, receptor tyrosine kinases; ERK, extracellular signal-regulated kinases; PI3K, phosphatidylinositol 3-kinase; AKT, murine thymoma viral oncogene homolog 1; PDGFRβ, platelet-derived growth factor receptor beta; IGF1R, insulin-like growth factor receptor; HGF, hepatocyte growth factor; HIF-1α, hypoxia-inducible factors-1alpha; mTOR, mechanistic target of rapamycin; EMT, epithelial-to-mesenchymal transition; p70S6K, ribosomal protein S6 kinase.

* Corresponding author. Dipartimento di Scienze Biomediche Sperimentali e Cliniche "Mario Serio", Università di Firenze, Viale G.B. Morgagni, 50, 50134 Firenze, Italy.
E-mail address: lido.calorini@unifi.it (L. Calorini).

[1] RJ and PS contributed equally to this manuscript.

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the over expression of COT (MAP3K8) that leads to ERK activation regardless of RAF signaling [11]. Also, the activation of the PI3K/AKT pathway in presence or in absence of MAPK reactivation drives vemurafenib resistance and this activation can be mediated by phosphatase and PTEN loss [12], or by the increase of RTK signaling [8]. Lastly platelet-derived growth factor receptor beta (PDGFRβ) and the insulin-like growth factor receptor (IGF1R) can be involved in the development of drug resistance [8,13].

Here, we proposed that a particular aspect of tumor microenvironment, that is a low extracellular pH, might participate to promote vemurafenib resistance in $BRAF^{V600E}$ melanoma cells.

Indeed solid tumors express acidic regions and that acidosis correlates with a poor prognosis and recurrence [14]. We know that tumor cells, even when there is enough O_2 to support mitochondrial function, use the so-called "aerobic glycolysis" which leads to the conversion of one molecule of glucose into 2 molecules of lactic acid and 2 H^+ to produce 2 ATP, compared to the 36 ATP produced by aerobic metabolism [15,16]. Aerobic glycolysis up-regulation in malignant phenotype, that is mostly due to stable genetic or epigenetic changes [15], is important for proliferating tumor cells since glucose can be converted to macromolecules (acetyl-CoA, glycolytic intermediates and ribose, fatty acids, nonessential amino acids and nucleotide biosynthesis) to be used for proliferation [17]. When oxygen tension reduces and a hypoxic microenvironment develops, stabilization of HIF-1α may regulate an elevated number of metabolic genes driving in tumor cells an anaerobic glycolysis pathway. So lactate and protons are produced in large excess either by aerobic and anaerobic glycolysis, and transported outside by a redundant families of lactate and H^+ transporters in order to maintain an intracellular pH compatible with survival and/or proliferation. Therefore, the extracellular pH of tumors turns to acidic and this acidity is also sustained by a poor blood perfusion and limited lymphatic vessels [18].

Acidosis is also linked with a multidrug resistance phenotype, so here we show that an acid microenvironment (pH 6.7 ± 0.1) may drive a vemurafenib resistance in melanoma cells carrying V600E mutation, disclosing a new subset of melanoma cells able to undergo tumor relapse after drug treatment. In addition, we have identified that resistance of acidic $BRAF^{V600E}$ melanoma cells may be overcome by everolimus treatment, a drug acting on mTOR activity.

Materials and methods

Cell lines and culture conditions

In this study, we used the melanoma cell line A375M6, isolated in our laboratory from lung metastasis of SCID bg/bg mice i.v. injected with A375 human melanoma cell lines, obtained from American Type Culture Collection (ATCC, Rockville, MD). In some experiments we used also the human melanoma cell lines WM266-4 (from ATCC), SkMel28 (from ATCC) and M21 (kindly provided by Dr. Antony Montgomery, The Scripps Research Institute, La Jolla, CA). Melanoma cells were cultivated in Dulbecco's Modified Eagle Medium high glucose (DMEM 4500, EuroClone, MI, Italy) supplemented with 10% fetal bovine serum (FBS, Boehringer Mannheim, Germany), at 37 °C in humidified atmosphere containing 90% air and 10% CO_2. Cells were harvested from subconfluent cultures by incubation with a trypsin-EDTA solution (EuroClone, MI, Italy), and propagated every three days. Viability of the cells was determined by trypan blue exclusion test. Cultures were periodically monitored for mycoplasma contamination using Chen's fluorochrome test.

Acidic treatment

Chemical acidified medium was obtained by the addition of HCl 1 N in DMEM 4500 10% FCS, pH value was monitored by a pH meter (Orion PH Meter 520A-1). As pH value was stable at 6.7 ± 0.1, acidified medium was added to cell cultures and the seal caps were tightly closed to prevent buffering. pH was evaluated at the end of experiment and found unchanged. Vemurafenib, trametinib and etoposide (MedChem Express, Stockholm Sweden) were added 24 h after the acidic treatment and used 0.5–5 μM, 100 nM and 100 μM, respectively; everolimus (MedChem Express, Stockholm Sweden) was added together with the acidic treatment and used 20 μM.

For transient acidosis, cells were grown in acidic medium for 24–72 h. For chronic acidosis, melanoma cells were cultured in pH 6.7 medium for approximately 3 months, until tumor cells recovered a similar growth rate as parent cells maintained at pH 7.4 [19]. During the long lasting acidic treatment, no significant death of cells was found.

Western blotting analysis

Cells were washed with ice cold PBS containing 1 mM Na4VO3, and lysed in 100 μL of cell RIPA lysis buffer (Merk Millipore, Vimodrone, MI, Italy) containing PMSF (Sigma-Aldrich), sodium orthovanadate (Sigma-Aldrich) and protease inhibitor cocktail (Calbiochem). Aliquots of supernatants containing equal amounts of protein (40 mg) in Laemmli buffer were separated on Bolt® Bis-Tris Plus gels 4–12% precast polyacrylamide gels (Life Technologies, Monza, Italy). Fractionated proteins were transferred from the gel to a PVDF nitrocellulose membrane using iBlot 2 system (Life Technologies, Monza, Italy). Blots were stained with Ponceau red to ensure equal loading and complete transfer of proteins, and then they were blocked for 1 h, at room temperature, with Odyssey blocking buffer (Dasit Science, Cornaredo, MI, Italy). Subsequently, the membrane was probed at 4 °C overnight with primary antibodies diluted in a solution of 1:1 Odyssey blocking buffer/T-PBS buffer. The primary antibodies were: rabbit anti-pAKT (1:1000, Cell signaling Technology, Danvers, MA, US), rabbit anti AKT (1:1000, Cell signaling Technology, Danvers, MA, US), rabbit anti-pERK (1:1000, Cell signaling Technology, Danvers, MA, US), rabbit anti-ERK (1:1000, Cell signaling Technology, Danvers, MA, US), mouse anti N-Cadherin (1:1000, DAKO, Glostrup, Denmark), mouse anti E-Cadherin (1:1000, DAKO, Glostrup, Denmark) rabbit anti p-p70S6K (1:1000, Cell signaling Technology, Danvers, MA, US) and rabbit anti p70S6k (1:1000, Cell signaling Technology, Danvers, MA, US). The membrane was washed in T-PBS buffer, incubated for 1 h at room temperature with goat anti-rabbit IgG Alexa Flour 750 antibody or with goat anti-mouse IgG Alexa Fluor 680 antibody (Invitrogen, Monza, Italy), and then visualized by an Odyssey Infrared Imaging System (LI-COR® Bioscience). Mouse anti-β-actin monoclonal antibody (1:2000, GeneTex Irvine, CA,USA) or mouse anti-α-tubulin monoclonal antibody (1:2000, Sigma, Saint Louis, MO, USA) were used to assess equal amount of protein loaded in each lane.

Proliferation assay

The proliferation was evaluated using CellTrace™ CFSE Cell Proliferation Kit (Life Technologies, Monza, Italy). Tumor cells were incubated for 20 min at 37 °C with the dye at the concentration of 5 μM; then the cells were cultured alone or with drugs. After that, the cells were detached, fixed in 5% paraformaldehyde and analyzed by flow cytometry. The fluorescence value obtained was analyzed by ModFit software to estimate the proliferation index. Proliferation assay was performed by growing cells in Dulbecco's Modified Eagle Medium high glucose supplemented with 10% fetal bovine serum at pH 7.4 or 6.7.

Cell cycle analysis

Cell cycle distribution was analyzed by the DNA content using propidium iodide (PI) staining method. Cells were centrifugated and stained with a mixture of 50 μg/ml PI (Sigma-Aldrich, St. Louis, Missouri), 0.1% trisodium citrate and 0.1% NP40 (or triton x-100) in the dark at 4 °C for 30 min. The stained cells were analyzed by flow cytometry (BD-FACS Canto) using red propidium-DNA fluorescence.

Evaluation of apoptosis

Apoptosis was measured by flow cytometry, using the Annexin V staining. Cells were washed once with PBS, detached with Accutase (Euroclone, MI, Italy), resuspended in 100 μL of 1x Annexin-binding buffer at the concentration of 1×10^6 cells/mL, stained with 5 μL of Annexin V FITC-conjugated (ImmunoTools, Friesoythe, Germany) and 1 μL of 100 μg/ml PI working solution and incubated at 4 °C in the dark condition for 15 min. Then, 400 μL of 1X Annexin Binding Buffer was added to each sample and cells were analyzed by flow cytometry (BD-FACS Canto) to find out the viability (annexin V and PI negative, Q3), early apoptosis (annexin V positive and PI negative, Q4), or late apoptosis (annexin V and PI positive, Q2). A minimum of 10000 events were collected.

Invasion assay

Cells invasion was studied in Boyden chambers in which the upper and lower wells were separated by 8 μm pore-size polycarbonate filters coated with Matrigel (12.5 μg/filter; BD Biosciences, Franklin Lakes, New Jersey). 1×10^5 cells suspended in 200 μl of their own growth medium were seeded in the upper compartment, while in the lower chamber fresh complete medium was added as chemo attractant. Cells were incubated for 6 h at 37 °C, 10% CO_2 in air, in the presence or absence of Ilomastat, a MMPs inhibitor (Millipore, Billerica, Massachusetts). After incubation, filters were removed and the non-invading cells on the upper surface were wiped-off mechanically with a cotton swab. Cells on the lower side of the filters were fixed overnight in ice-cold methanol, then stained using a DiffQuick kit (BD Biosciences) and pictures of randomly chosen fields were taken.