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Original Articles

The repopulating cancer cells in melanoma are characterized by increased mitochondrial membrane potential

Don G. Lee [a,b,c,1], Beom K. Choi [b,1], Young H. Kim [a,b], Ho S. Oh [a,b,c], Sang H. Park [b,c], Young Soo Bae [c], Byoung S. Kwon [a,d,*]

[a] T Cell Therapy Unit, Eutilex Research Institute of Biomedicine, 222 Banpo-daero, Seocho-gu, Seoul, Republic of Korea
[b] Cancer Immunology Branch, Division of Cancer Biology, National Cancer Center, Goyang, Republic of Korea
[c] Department of Biological Sciences, Sungkyunkwan University, Suwon, Republic of Korea
[d] Section of Clinical Immunology, Allergy, and Rheumatology, Department of Medicine, Tulane University Health Sciences Center, New Orleans, LA, USA

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A B S T R A C T

Although considerable effort has been expended in identifying definitive markers for cancer stem cells (CSCs) or cancer-initiating cells (CICs), the phenotypic plasticity of these cells obviates simple characterization using cell surface markers. We hypothesized that these cells could be characterized by their metabolic properties because they are in a quiescent state with low energy needs. We examined whether cancer cells differ in mitochondrial membrane potential (Δψm) when they are under stress. The Δψm of B16-F10 melanoma cells increased when they were exposed *in vitro* to serum starvation and chemotherapeutic agents, but not when exposed to hypoxia. Such $TMRE^{high}$ cells were also present in tumor tissue. They primarily used glucose and/or lactate, and were superior to $TMRE^{low}$ B16-F10 cells in their ability to drive tumor growth. These findings suggest that CSCs or CICs could be identified in heterogeneous melanoma populations by measuring Δψm.

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Introduction

The cancer stem cell (CSC) or cancer initiating cells (CIC) hypothesis postulates that a minor subpopulation within tumors drives tumor initiation, sustains tumor growth, and has functional traits corresponding to tissue stem cells [1]. These cancer cells can self-renew, induce tumorigenesis, and facilitate relapse and metastasis by seeding new tumors. Several studies report that aggressive and poorly differentiated tumor cells have transcriptional signatures that are similar to those of embryonic stem cells (ESCs), which include transcripts of SOX2, OCT3/4, NANOG, and Nestin [2,3].

Moreover, genetic labeling techniques applied to mouse models of skin, intestine and brain tumors have provided strong evidence for the existence of CSCs *in vivo* [4–6]. The CSCs appear to originate from normal somatic stem cells through genetic deregulation. Another possibility, though, is that they are somatic cancer cells that have acquired stem cell-like features due to genetic mutations and environmental effects such as hypoxia, starvation and inflammation. However, other workers have argued that the plasticity of cancer cells allows cells of the bulk proliferating cancer population to develop CSC- or CIC-like properties by acquiring particular epigenetic and transcriptional signatures [7,8]. If that is so, CSCs or CICs are simply cancer cells in a reversible dynamic state of differentiation rather than a specific cancer subpopulation.

Metastatic melanoma is one of the most aggressive skin cancers, with poor survival rates [9]. CD20, ABCB5, CD133 and CD271 are reported to be markers for CSCs in human melanoma, and melanoma cells positive for these markers promoted tumor growth when xenografted into immune-deficient NOD/SCID, NSG or $RAG2^{-/-}\gamma c^{-/-}$ mice [10–13]. However, according to other work, melanomas are not stably hierarchically organized; instead their heterogeneity results from reversible phenotypic changes within tumor tissue [14]. This would imply that tumorigenic and non-tumorigenic melanoma cells could not be identified by particular markers, and that large proportion of melanoma cells would be tumorigenic and have features of CSC-like or tumor-initiating cells.

If melanoma CSCs represent reversible cellular states within the tumor microenvironment, the melanoma CSC cannot be simply characterized by cell surface markers. Rather, CSCs need to be characterized by their metabolic properties because CSC-like properties

Abbreviations: CSC, cancer stem cell; TMRE, tetramethylrhodamine ethyl ester; Δψm, mitochondrial membrane potential; ESCs, embryonic stem cells; MTG, mitotracker green FM; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; BPTES, bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide; ETX, etomoxir; 2-DG, 2-deoxy-D-glucose; CTX, cyclophosphamide; MTX, methotrexate.

* Corresponding author. Fax: +82 2 532 0630.
E-mail address: bskwon@eutilex.com (B.S. Kwon).

[1] These authors contributed equally to this work.

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will be maintained by finely tuning their metabolic activities. We suspected that cancer cells with stemness are quiescent with low energy demands but that they might have elevated mitochondrial mass and/or membrane potential (Δψm) to actively differentiate and proliferate after recovery from stressful conditions. Therefore, we measured the mitochondrial mass and Δψm of B16-F10 mouse melanoma cells that survived various stress conditions, and examined whether the changes in mitochondrial mass and Δψm could be used to discriminate the CSC- or CIC-like cells within melanoma populations.

Materials and methods

Mice, reagents, and antibodies

Female C57BL/6 mice aged six-to-eight-weeks were purchased from OrientBio (Gapyeong, Korea). All animal experiments were reviewed and approved by the Animal Care and Use Committee of the National Cancer Center (NCC-10-080), and were performed in accordance with the Guide for the Care and Use of Laboratory Animals. MitoTracker Green FM (MTG) was purchased from Cell Signaling (Danvers, MA), tetramethylrhodamine ethyl ester (TMRE) was from Life Technologies (Carlsbad, CA), and 7-AAD was from BD Pharmingen (San Diego, CA). FITC-anti-CD133 and PE-anti-CD44 were purchased from eBioscience (San Diego, CA), and FITC-anti-CD24 from Abcam (Cambridge, MA). Carbonyl cyanide m-chlorophenyl hydrazine (CCCP), oligomycin A, DMSO, L-lactic acid, bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl) ethyl sulfide (BPTES), etomoxir (ETX), and 2-deoxy-D-glucose (2-DG) were purchased from Sigma-Aldrich (St. Louis, MO). Cisplatin was purchased from Dong-A Pharmaceutical CO., LTD (Seoul, Korea) and cyclophosphamide (CTX) from JW Pharmaceutical CO., LTD (Seoul, Korea). Methotrexate (MTX) was obtained from Hospira (Korea). Stock solutions of MTG (10 μM in DMSO), TMRE (10 μM in DMSO), CCCP (20 mM in DMSO), oligomycin (1 mg/ml in DMSO), L-lactic acid (2 M in water), BPTES (10 mM in DMSO), ETX (10 mM in water), 2-DG (1 M in water), cisplatin (0.5 mg/ml), CTX (20 mg/ml), and MTX (25 mg/ml) were stored at 4 °C or –20 °C.

Cell culture and treatments

B16-F10 mouse melanoma cells were purchased from the ATCC (acquired in 2009) and authenticated with an Identifiler® PCR Amplification Kit (Life Technologies, March 2014). B16-F10 cells were maintained in high glucose DMEM medium (Welgene) supplemented with 10% FBS (CellGro) and penicillin/ streptomycin (Gibco) at 37 °C with 5% CO_2. B16-F10 cells were plated at 1×10^5 cells/ml in 6-well plates in high glucose DMEM medium containing 0.5, 1, 10% FBS and then treated with the chemotherapeutic drugs cisplatin (5 or 10 μg/ml), CTX (1 or 2 mg/ml), and MTX (20 or 50 nM). All cultures were maintained at 37 °C in a humidified chamber with 5% CO_2. After 3 days of culture, viable cells were counted using an ADAM MC automated mammalian cell counter (Bulldog Bio Inc., Rochester, NY). To evaluate the impact of glucose and lactic acid on cancer cell survival, B16-F10 cells were cultured in DMEM medium with and without glucose (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% or 0.5% FBS, and incubated w/ or w/o L-lactic acid (Sigma-Aldrich, St. Louis, MO) for 3 days. To evaluate the effect of metabolic inhibitors on Δψm, B16-F10 cells were treated with 0, 1, 3 or 5 mg/ml oligomycin, or 1, 10 or 20 μM CCCP. The cells were cultured in medium containing 0.5% or 10% FBS in the presence of 5 or 10 μM BPTES, 100 or 200 μM ETX, and 1, 5 or 10 mM 2-DG for 3 days. Hypoxic conditions were achieved using an Invivo2 hypoxia workstation (Ruskin Technologies) according to the manufacturers' instructions. Cells were cultured in 1% O_2, 5% CO_2 and 94% N_2 for 1 or 2 days.

Mitochondrial mass and membrane potential

B16-F10 cells were suspended in culture medium at 1×10^6 cells/ml, stained with 100 nM MTG for 15 min at 37 °C, washed with PBS twice, and incubated with 60 nM TMRE for another 15 min at 37 °C. After washing with PBS twice, the cells were stained with 7-AAD and subsequently analyzed by FACSCalibur (BD Bioscience).

Tumor challenge

Serum-starved B16-F10 cells or single cell suspensions of B16-F10 tumor tissue (post-injection day 14) were stained with 100 nM MTG for 15 min at 37 °C. They were washed twice with PBS and incubated with 60 nM TMRE for 15 min at 37 °C, washed again with PBS twice, and stained with 7-AAD. $TMRE^{high}$ and $TMRE^{low}$ cells were separated with a FACSAria™ (BD Biosciences). The sorted B16-F10 cells were subcutaneously (s.c.) injected into the backs of 6-wk-old female B6 mice at doses of 1×10^3, 1×10^4 and 1×10^5 cells per mouse. The mice were monitored daily for tumor growth and survival.

Results

B16-F10 melanoma cells that survive serum starvation possess increased mitochondrial mass and membrane potential

The tumor microenvironment generates multiple cellular stresses ranging from hypoxia and low nutrient availability, to immune infiltration. These conditions alter cell metabolism and affect mitochondrial function, thereby exacerbating tumor aggressiveness and complicating therapeutic interventions [15,16]. Therefore, we examined whether mimicking the tumor microenvironment by imposing serum starvation/hypoxic conditions *in vitro* would alter mitochondrial functions. First we determined whether the mitochondrial mass and Δψm of B16-F10 melanoma cells was altered by serum starvation. B16-F10 cells were cultured in DMEM medium containing 0.5, 1, and 10% FBS for 3 days. By day 3 numbers of live cells had declined in 0.5 and 1% FBS (Fig. 1A). The cultured cells were stained with MTG to measure mitochondrial mass and with TMRE to assess Δψm; they were also stained with 7-AAD to exclude dead cells. Flow cytometric analysis demonstrated that the mitochondrial mass and Δψm of the surviving cells increased (Fig. 1).

Since hypoxia is another factor affecting the behavior of tumor cells, we next examined whether hypoxia changed the Δψm of B16-F10 cells. B16-F10 cells were cultured in normal or 1% O_2 for 1 or 2 days, and stained with MTG, TMRE and 7-AAD. Flow cytometric analysis indicated that mitochondrial mass and Δψm did not increase under the hypoxic condition on day 1, and cells with high Δψm actually declined by day 2 (Fig. 1D and E). These results indicate that the melanoma cells that survive serum starvation have increased Δψm but the same is not true for survivors of anoxia.

Chemotherapeutic treatment increases the Δψm of melanoma cells

Since the Δψm of B16-F10 melanoma cells increased under serum starvation, we next asked whether it would increase in response to treatment with chemotherapeutic agents. B16-F10 cells were treated with cisplatin, cyclophosphamide (CTX) or methotrexate (MTX), and mitochondrial mass and Δψm were assessed. First, B16-F10 cells were cultured in the presence of 0, 5 and 10 μg/ml of cisplatin for 2 days. We found that cisplatin made the B16-F10 cells more adherent and markedly reduced viable cells (Fig. 2A and B). Flow cytometric analyses demonstrated that their Δψm (Fig. 2C; middle panel) and granularity (Fig. 2C; far left panel) increased.

The Δψm of B16-F10 cells that survived treatment with CTX or MTX was similarly enhanced (Fig. 2D and E). Thus these results suggest that elevated Δψm may serve as a general sign that B16-F10 melanoma cells have survived stressful conditions.

B16-F10 melanoma cells with elevated Δψm have enhanced repopulating ability in vivo

Although we found that the Δψm of B16-F10 melanoma cells increased following serum starvation or chemotherapeutic treatments, it was not clear whether the augmented Δψm was required for survival of the cells under such stressful conditions. To test this, B16-F10 cells were pre-incubated with 10 μM CCCP, a chemical inhibitor of oxidative phosphorylation, and cultured in serum-starved conditions or in the presence of CTX or cisplatin for 2 days. 10 μM CCCP had a minimal effect on the survival of B16-F10 cells in 10% FBS, but significantly reduced their viability when serum-starved or in the presence of CTX or cisplatin (Fig. 3A and B). This suggests that Δψm plays a pivotal role in the survival of tumor cells under challenging conditions.

Since serum starvation or chemotherapeutics treatment resulted in B16-F10 cells with low and high levels of Δψm all of whom

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