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Cancer cells recovering from damage exhibit mitochondrial restructuring and increased aerobic glycolysis

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ABSTRACT

Instead of relying on mitochondrial oxidative phosphorylation, most cancer cells rely heavily on aerobic glycolysis, a phenomenon termed as "the Warburg effect". We considered that this effect is a direct consequence of damage which persists in cancer cells that recover from damage. To this end, we studied glycolysis and rate of cell proliferation in cancer cells that recovered from severe damage. We show that *in vitro* Damage-Recovered (DR) cells exhibit mitochondrial structural remodeling, display Warburg effect, and show increased *in vitro* and *in vivo* proliferation and tolerance to damage. To test whether cancer cells derived from tumor microenvironment can show similar properties, we isolated Damage-Recovered (T^{DR}) cells from tumors. We demonstrate that T^{DR} cells also show increased aerobic glycolysis and a high proliferation rate. These findings show that Warburg effect and its consequences are induced in cancer cells that survive severe damage.

1. Introduction

Most cancer cells utilize aerobic glycolysis for their energy needs (Warburg effect), despite the fact that this pathway is an inefficient way to generate adenosine 5′-triphosphate (ATP) when oxygen is abundant [1–3]. It is still not well understood as why such a less efficient metabolism is selected by proliferating cancer cells. One reasonable explanation would be that inefficient ATP production happens only when energy resources are scarce, such as in areas of the tumor where low blood flow leads to low nutrient, hypoxic and an acidotic conditions [4]. However, some cancer cells appear to rely on glycolytic metabolism even before they are exposed to such harsh conditions [5–8]. Some consider that the adoption of aerobic glycolysis by cancer cells is driven by tumor hypoxia [9] while others consider that aerobic glycolysis might be multi-factorial and driven by oncogenes, tumor suppressors, mtDNA mutations and other factors [9–11].

In this study, we aimed to address whether the Warburg effect might be a consequence of damage which occurs in hypovascularized regions of tumors that create hypoxia, glucopenia and acidosis. We demonstrate here that cancer cells that recover from such damages exhibit mitochondrial structural changes including development of a primordial type as evidenced by ballooning and remodeling of cristae. These changes are associated with decreased copy number of mtDNA, increased expression of glycolytic enzymes, activation of glycolytic system of energy production, increased ATP synthesis, increased tolerance to damage as well as rapid *in vitro* and *in vivo* proliferation. We also show that cells that recover from damage endured *in vivo* are also highly glycolytic and exhibit a high rate of cell proliferation. These findings suggest a role for damage-recovery in the adoption of "Warburg effect" by the tumor cells. Furthermore, these data indicate that cancer cells that emerge after recovery from damage, display new metabolic characteristics consistent with a higher degree of malignant behavior as evidenced by damage tolerance and increased rate of cell proliferation.

2. Materials and methods

2.1. Reagents and cell culture

Chemicals were from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Trypan Blue was purchased from

Abbreviations: Pc, parental control; DR, Damage-Recovered; T^{V}, Viable tumor derived cells; T^{DR}, Damage-Recovered tumor derived cells; ECAR, extracellular acidification rates; ATP, adenosine 5′-triphosphate; AUC, area under the curve; BrdU, 5-bromo-2′-deoxyuridine; *CYT B* and *C*, *CYTOCHROME B* and *C*; 2DG, 2-deoxyglucose; *ENTPD5*, ectonucleoside triphosphate diphosphohydrolase 5; FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; *HK1*, *HEXOKINASE 1*; *LDH A*, lactate dehydrogenase A; *mtTFA*, mitochondrial transcription factor A.

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Sigma-Aldrich and staining was performed as described by the manufacturer. Collagenase-P was purchased from Roche Applied Science (Indianapolis, IN). RNAlater™ RNA Stabilization Reagent and RNeasy Mini Kit for total RNA isolation were purchased from Qiagen Inc. (Valencia, CA). The antibodies were from Millipore (Billerica, MA), Invitrogen (Carlsbad, CA) and Jackson ImmunoResearch Laboratories (Baltimore, PA). CT26 mouse colon carcinoma cell line (CRL-2639) was obtained from ATCC (Manassas, VA), and maintained in RPMI 1640 with 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 25 mM glucose, 1.5 g/L sodium bicarbonate and 10% fetal bovine serum in 37 °C incubator with 5% CO_2. Condition of damages were as follows; for induction of heat induced damage (DR^{He}), cells were incubated at 45 °C for 45 min, for induction of hypoxic damage (DR^{H}), cells were incubated in 0.3% O_2 at 37 °C, and for damage induced by glucose deprivation (DR^{G-}), cells were cultured in a medium with no glucose in 5% CO_2 at 37 °C. For induction of damage by acidosis (DR^{A}), cells were cultured in RPMI-based medium, pH was adjusted to 6.0 and cells were incubated in 5% CO_2 at 37 °C. Cultures were subjected to hypoxia, glucose deprivation, and acidosis for 3–5 days until majority of cells were lost due to cell death.

2.2. Animal experiments and isolation of cancer cells derived from in vivo grown tumors

Animal care and all procedures carried out on animals were approved by the Institutional Animal Care Committees at University of California, Irvine. Each athymic nude (NOD/SCID) mouse received 10^5 cancer cells in a total volume of 200 µl subcutaneously in four locations in the mid-abdominal and lower flank areas after anesthesia. Tumors were removed and weighed. Tumors were minced into <1 mm blocks, and incubated with 250 µg/ml Collagenase-P at 37 °C for 20 min. Suspension of single tumor cells were >95% viable (T^{V}) as determined by Trypan blue staining. Sedimented small fragments that settled to the bottom of the tubes were comprised of apoptotic regions with large areas of Trypan blue positive cells. These fragments were subjected to treatment with 0.25% trypsin at 37 °C for 10 min. Majority (>95%) of single cells in the cell suspension after incubation were Trypan blue positive. This cell suspension was cultured for 24 h to allow viable cells to bind to the culture substrate. The floating cells were subsequently transferred weekly to new culture vessels. Damaged cells that recovered (T^{DR}) and started to proliferate were collected after 2 weeks.

2.3. Immunostaining

Cells were fixed for 5 min in 2% buffered paraformaldehyde containing 0.02% NP40, and subjected to blocking for 10 min in 2% serum. For γH_2AX staining, cells were incubated with anti-γH2AX antibody conjugated with biotin (1–2 µg/ml; Millipore) followed by Peroxidase-Streptavidin (0.1 µg/ml; Jackson ImmunoResearch). The γH2AX staining was developed in 3,3′-diaminobenzidine (DAB)-H_2O_2 (DAKO; Carpinteria, CA) and viewed at the light microscopic level without counterstain. For BrdU staining, cells were incubated with anti-BrdU antibody conjugated with biotin (1–2 µg/ml; Invitrogen) followed by Tetra-methyl Rhodamine-Streptavidin (0.1 µg/ml; Jackson ImmunoResearch). The fluorescent images were

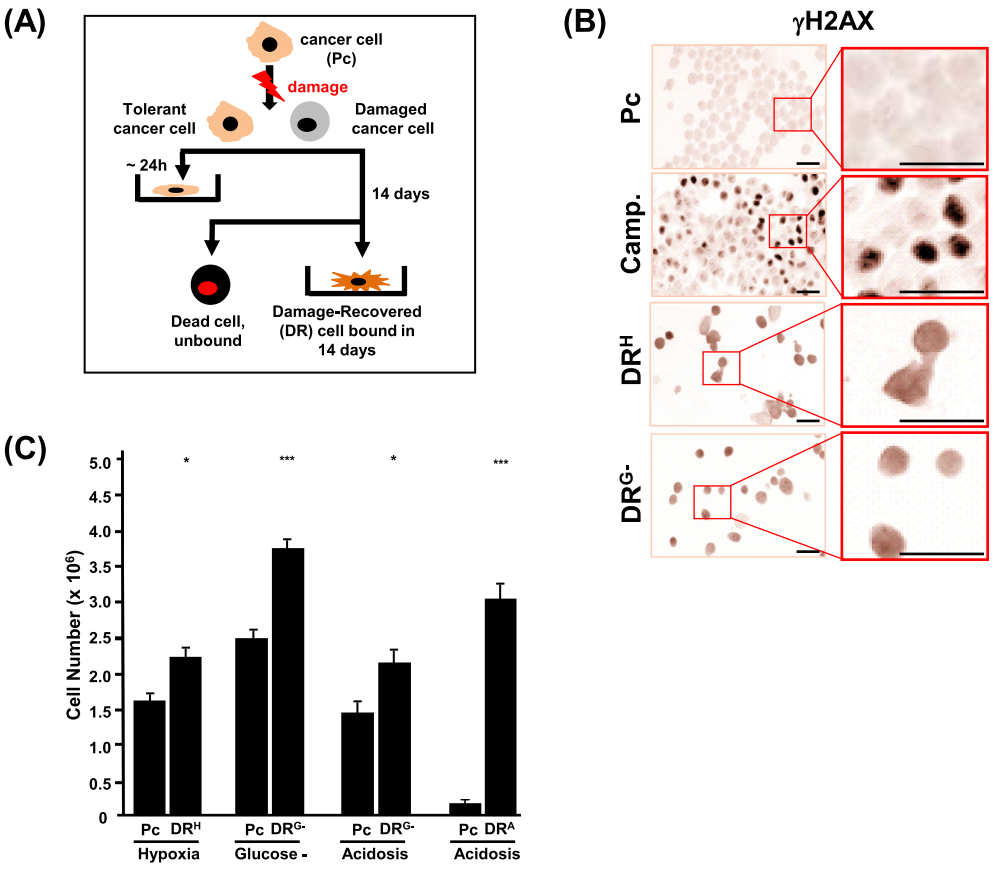

Fig. 1. Damage-Recovered (DR) cells show increased tolerance to damage. (A) Scheme for isolation of DR cells after damage by heat (DR^{He}), hypoxia (DR^{H}), glucose deprivation (DR^{G-}) and acidosis (DR^{A}). (B) Immunostaining of γH2AX in Pc, camptothecin-treated Pc, DR^{H} and DR^{G-} CT26 cells. Scale bars, 50 µM. (C) Number of DR cells remaining after being subjected to hypoxia, glucose deprivation and acidosis after 5 days. Culture dishes were seeded with 2.5×10^6 cells in triplicate and the number of viable cells was determined by Trypan blue staining. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.