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A bioenergetic profile of non-transformed fibroblasts uncovers a link between death-resistance and enhanced spare respiratory capacity☆

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ABSTRACT

Apoptosis-resistance and metabolic imbalances are prominent features of cancer cells. We have recently reported on populations of human fibroblasts that exhibit resistance to mitochondrial-mediated apoptosis, acquired as a result of a single genotoxic exposure. The objective of the present study was to investigate the intrinsic bioenergetic profile of the death-resistant cells, as compared to the clonogenic control cells. Therefore, we analyzed the basic bioenergetic parameters including oxygen consumption and extracellular acidification rates, coupling efficiency, and spare respiratory capacity. Our data demonstrate a strong correlation between enhanced spare respiratory capacity and death-resistance, which we postulate to be indicative of the earliest stages of carcinogenesis.

1. Introduction

Several lines of evidence have indicated that carcinogenesis evolves from consecutive genetic and/or epigenetic alterations that provide cellular survival advantages, ultimately leading to the conversion of normal human cells to malignant cancer cells [for review, see (Hanahan and Weinberg, 2000, 2011)]. As evasion of apoptosis and unlimited replicative potential are hallmarks of cancer, two questions become inevitable when studying death-resistance and early-stage carcinogenesis: 1) how does a cell gain enhanced proliferative capacity, and 2) what fuels this unlimited replicative potential? In most cells under aerobic conditions, glucose is converted to pyruvate through glycolysis, which then enters the TCA cycle where the flavin nucleotide ($FADH_2$) and NADH are produced. The respective reduced equivalents are further oxidized by the mitochondrially-localized electron transport chain (ETC), which ultimately produces ~80% of total cellular ATP. Conversely, early studies in tumor cells have found an upregulation of glycolysis for energy production, even in the presence of sufficient oxygen levels. This phenotype is known as the Warburg effect, after Dr. Otto Warburg who uncovered the phenomenon (Warburg, 1956). While the high glycolytic phenotype of cancer cells has been exploited for clinical use (i.e. positron emission tomography), the molecular basis of the Warburg effect remains unclear.

The bioenergetic profile of several cell types such as neurons, endothelial cells, and human carcinoma cell lines, has been characterized in an effort to uncover alterations that may be targeted for therapeutic purposes (Rodriguez-Enriquez et al., 2008; van der Windt et al., 2012; Wu et al., 2007; Xun et al., 2012). Of particular interest to the present study, is the role that spare respiratory capacity (SRC) may play in death resistance. The term, SRC, describes the reserve capacity that enables the production of energy in response to cellular stress (Nicholls, 2009). It has been hypothesized that in the face of oxidative stress, cell survival can be potentiated when a maximal reserve of ATP is maintained (Choi et al., 2009; Fern, 2003; Zhu et al., 2012).

We have previously generated sub-populations of BJ-hTERT human diploid foreskin fibroblasts, which have acquired resistance to cell death induced by hexavalent chromium [Cr(VI)], a broad-spectrum DNA-damaging agent (Pritchard et al., 2005). Fibroblasts are integral to the cellular microenvironment and have been associated with pathological conditions such as fibrosis and carcinogenesis (McAnulty, 2007; Vaheri et al., 2009). This system is unique in that it models initial molecular events that occur in a normal cell that survived a single, acute, initiating genotoxic challenge. While the selection model in this study was

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generated by Cr(VI) treatment, it also exhibits a cross-resistance to the well-known chemotherapeutic agent, cisplatin, as well as to H_2O_2 (Nickens et al., 2012). Long-term exposure to certain forms of Cr(VI) is associated with respiratory carcinogenesis (IARC, 1990). Our recent report investigated the death-sensitivity of subclonal populations derived from clonogenic survivors of BJ-hTERT cells treated with Cr(VI) (DR), or selected by dilution-based cloning without treatment (CC) (Nickens et al., 2012). Notably, our data suggested the presence of more resilient mitochondria in DR cells, and that death resistance can be acquired in normal human cells early after genotoxin exposure. Taken together, these data led us to postulate that resistance to mitochondrially-mediated cell death and mitochondrial dysregulation may be initial phenotypic alterations associated with early-stage carcinogenesis.

Here we report on the bioenergetic profile of the DR and CC subclonal cell lines. By employing the Seahorse Bioscience XF Extracellular Flux Analyzer, we simultaneously measured glycolysis by assessing the extracellular acidification rate (ECAR), as well as the rate of oxidative phosphorylation by measuring the cellular oxygen consumption rate (OCR) (Eklund et al., 2004). We tested the hypothesis that survival after genotoxic stress may involve the selection of cells with intrinsically altered bioenergetic regulation. Our data show that while there is no difference in basal ATP content, ECAR, or OCR, there is an increase in the SRC of the DR cells, as compared to the CC cells. Taken together, the present data show that a greater intrinsic SRC is coincident with death-resistance in our model system. Moreover, this enhanced capacity may be a mechanistic step in the acquisition of death-resistance, which in turn may potentially foster neoplastic progression. Importantly, we show that the intrinsically enhanced SRC was observed in diploid human cells that have acquired a death-resistant phenotype following only a single exposure to a carcinogen.

2. Materials and methods

2.1. Subcloning, cell lines, and culture parameters

Subclonal populations were derived as previously described (Nickens et al., 2012). The cell lines used in the present study include untreated clonogenic control cell lines, CC1 and CC2, as well as clones derived from clonogenic survivors of Cr(VI) exposure, DR1, DR2, DR3, and DR4, which display an apoptosis resistant phenotype. As previously reported, all CC and DR cell lines were derived from human foreskin fibroblasts transfected with the hTERT gene (BJ-hTERT; Geron Corp.), and further subcloned (at passage 138) into both CC and DR cell lines (Nickens et al., 2012; Pritchard et al., 2005). The original hTERT-immortalized foreskin fibroblast cell line, BJ-5ta, was derived by transfecting the BJ foreskin fibroblast cell line with the pGRN145 hTERT-expressing plasmid (ATCC MBA-141) (Bodnar et al., 1998). The population doubling time of the respective death resistant and death sensitive sub-populations is similar, at around 22–26 h. For the present study, cells were passaged at 80% confluence, and were only used for 10 passages, before a fresh vial was selected.

All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen Corporation, Carlsbad, CA), containing medium 199 (Invitrogen Corporation) (4:1), 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 5 μg/ml gentamicin (Life Technologies, Gaithersburg, MD), and 0.75 μg/ml Fungizone antimycotic (Invitrogen Corporation; DMEM complete). The hTERT transgene was selected for by the addition of 10 μg/ml hygromycin B (Life Technologies) to the medium. All cell lines were incubated in a 95% air and 5% CO_2 humidified atmosphere at 37 °C, and the medium was replaced every 48 h.

2.2. ATP content

Cells were seeded at a density of 4×10^5/60 mm dish and incubated at 37 °C for 24 h prior to analysis. Following incubation, the cells were rinsed with 5 ml PBS and removed from the dish by gentle scraping in 60 μl of CHAPS cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing 50 mM PIPES/HCl (pH 6.5), 2 mM EDTA, 0.1% CHAPS, 20 μg/ml leupeptin, and 10 μg/ml aprotinin; supplemented with 50 mM NaF, 1 mM Na_3V0_4, and 1 mM PMSF. The cells were lysed on ice, and the lysates were centrifuged at 14,000 rpm for 12 min at 2–8 °C. A luciferin–luciferase based bioluminescent ATP determination kit (Molecular Probes) was used to analyze ATP content (Ahn et al., 2008), according to the manufacturer's instructions. Briefly, ATP standards ranging from 0.5 to 25 μM were prepared from a 5 mM ATP stock solution in dH_2O. Duplicate aliquots of standards and samples were pipetted into a flat-bottom black polystyrene 96-well assay plate (Corning Incorporated, Corning, NY). The reaction was started immediately before analysis by the addition of the standard reaction solution containing 20× reaction buffer, 0.1 M DTT, 10 mM D-luciferin, and 5 mg/ml firefly luciferase. Luminescence was measured after a 200 ms integration time using 485 nm excitation and 538 nm emission wavelengths on the Fluoroskan Ascent FL (Thermo Fisher Scientific, Waltham, MA), followed by analysis using the Ascent Software (Ascent Software, London, UK). Background luminescence was subtracted from the generated values using wells containing only the standard reaction solution. Luminescence was normalized to protein content which was assessed using the BCA protein assay kit (Thermo Fisher Scientific).

2.3. Metabolic flux analysis

Cells were seeded in triplicate in 100 μl DMEM complete medium at a density of 1×10^4/well in XF24 24-well V7 cell culture plate (Seahorse Bioscience, North Billerica, MA), leaving appropriate temperature control wells empty, and incubated at 37 °C for 1 h. Following cell attachment, an additional 150 μl of medium was added to each well, and incubated at 37 °C overnight. The XF24 sensor cartridge (Seahorse Bioscience) was prepared by incubation of each sensor pair in 1 ml of Seahorse Bioscience XF24 calibrant pH 7.4 (Seahorse Bioscience) at 37 °C without CO_2 for 24 h. Prior to analysis, the medium was gently removed from the adherent cells and the wells were washed with 1 ml of pre-warmed specially formulated Seahorse DMEM (Seahorse Bioscience) supplemented with 25 mM glucose (assay medium). A final volume of 450 μl assay medium was added to each well and the plate was incubated at 37 °C without CO_2 for 1 h. Either 1.5 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) or 5 μM oligomycin (Sigma-Aldrich, St. Louis, MO) was added to injection port A of the XF24 sensor cartridge in assay medium and equilibrated at least 15 min prior to analysis at 37 °C without CO_2. The XF24 sensor cartridge was calibrated in the Seahorse XF24 analyzer (Seahorse Bioscience), which was pre-warmed to 37 °C. Following calibration, the cell culture plate was placed in the analyzer and the extracellular acidification rate (ECAR) and the oxygen consumption rates (OCR) were simultaneously measured via the following protocol: three cycles of mix (2 min) and delay (2 min); seven cycles of measure (4 min), mix (2 min) and delay (2 min); port A injection; six cycles of mix (2 min), delay (2 min) and measure (4 min). After the final measurement the assay medium was removed from each well and the cells were washed twice with warm PBS then incubated at 37 °C without CO_2 in 4 μM calcein AM (BD Biosciences, San Jose, California) in PBS for 40 min. Fluorescence was measured by using 485 excitation and 530 nm emission wavelengths on the Microplate Reader-Infinite® M1000 (Tecan Group Ltd., Männedorf) followed by data collection on the Tecan i-control software (Tecan Group Ltd.). ECAR and OCR rates were normalized to cell number as assessed by calcein AM fluorescence. Basal values were taken from the last measurement prior to drug injection and the post-drug injection values were from the first measurement after injection. Post-drug injection values were normalized to respective basal value and expressed as either mpH/min/10^4 cells (ECAR) or pmoles/min/10^4 cells (OCR). Cell number was determined by calcein AM incorporation (Zhang et al., 2012).