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Original Article

GOT1-mediated anaplerotic glutamine metabolism regulates chronic acidosis stress in pancreatic cancer cells

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ABSTRACT

The increased rate of glycolysis and reduced oxidative metabolism are the principal biochemical phenotypes observed in pancreatic ductal adenocarcinoma (PDAC) that lead to the development of an acidic tumor microenvironment. The pH of most epithelial cell-derived tumors is reported to be lower than that of plasma. However, little is known regarding the physiology and metabolism of cancer cells enduring chronic acidosis. Here, we cultured PDAC cells in chronic acidosis (pH 6.9–7.0) and observed that cells cultured in low pH had reduced clonogenic capacity. However, our physiological and metabolomics analysis showed that cells in low pH deviate from glycolytic metabolism and rely more on oxidative metabolism. The increased expression of the transaminase enzyme *GOT1* fuels oxidative metabolism of cells cultured in low pH by enhancing the non-canonical glutamine metabolic pathway. Survival in low pH is reduced upon depletion of *GOT1* due to increased intracellular ROS levels. Thus, *GOT1* plays an important role in energy metabolism and ROS balance in chronic acidosis stress. Our studies suggest that targeting anaplerotic glutamine metabolism may serve as an important therapeutic target in PDAC.

Introduction

Metabolic alterations represent an important hallmark of cancer cells [1]. Metabolic reprogramming allows cancer cells to sustain uncontrolled proliferation by rapid generation of ATP, biosynthesis of macromolecules, and maintenance of redox status [2]. Cancer cells can also reprogram the major metabolic pathways (carbohydrates, proteins, lipids, and nucleic acids) to meet these basic demands for uncontrolled proliferation [3,4]. The characteristic metabolic phenotype seen in cancer cells is the Warburg effect, which operates by enhancing glucose uptake and flux into glycolysis, while simultaneously diminishing the glucose carbon flux that enters the TCA cycle in the mitochondria, even in the presence of oxygen [5,6]. Although ATP generation through substrate level phosphorylation is very rapid, this mechanism is far less efficient than oxidative phosphorylation in generating energy from glucose. Thus, the metabolic phenotype observed in the Warburg effect demands very high glucose uptake to meet the energetic, biosynthetic, and redox needs of cancer cells. For these reasons, the increased glucose uptake of cancer cells is useful for diagnosing cancer using radiolabeled glucose analog ^{18}F-fluorodeoxyglucose and positron emission tomography (FDG-PET) to image and evaluate tumor progression without the need of a biopsy [7,8].

Because of the enhanced metabolic rate of rapidly proliferating tumor cells, the glucose that is metabolized through substrate level phosphorylation produces lactic acid as the end product. Lactic acid is a weak acid, and it quickly dissociates and loses a hydrogen ion to produce lactate [9]. Lactate is transported outside of the cell by monocarboxylate symporters along with protons resulting in decreased pH in the extracellular milieu [10,11]. Intracellular hydrogen ions can also be removed by sodium hydrogen exchangers that import sodium ions and extrude hydrogen ions, thereby acidifying the extracellular environment [12,13]. Similarly,

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vacuolar ATPases extrude hydrogen ions against their concentration gradient to the extracellular space, and hence, lower the extracellular pH [14]. *In vitro* studies have shown that rapidly growing cells, which exhibit the Warburg effect, increase the expression of these cell surface proteins to maintain an alkaline intracellular pH environment [15,16]. Indeed, increased intracellular pH is an established permissive signal for cellular proliferation promoting survival by limiting apoptosis, a process that is associated with intracellular acidification [17,18]. The role of low extracellular pH in carcinogenesis is thus paradoxical: on one hand, alkaline intracellular pH promotes proliferation and survival, while at the same time, low extracellular pH promotes invasion and metastasis at the cost of inducing stress, senescence, and apoptosis [12,19,20].

In addition to glucose, glutamine metabolism is also essential for the proliferation of cancer cells. Recent studies have demonstrated that glutamate derived from glutamine is utilized by highly proliferative cells to generate non-essential amino acids (NEAAs) through the glutamic-oxaloacetic transaminase enzymes (*GOT1* and *GOT2*), while quiescent cells metabolize glutamate through *GLUD1* (glutamate dehydrogenase 1) and subsequent decarboxylation reactions in the TCA cycle [21,22]. Thus, glutamine can be metabolized through both anabolic (anaplerotic) and catabolic pathways.

Several oncogenes are implicated in reprogramming tumor cell metabolism. One such gene is *KRAS,* which upon accumulating activating mutations serves as a key signature oncogene that plays a prominent role in malignant transformation and tumor progression in PDAC [23,24]. PDAC cells with oncogenic *KRAS* have reprogrammed glucose and glutamine metabolism to serve anabolic processes [25,26]. Canonical glutamine metabolism occurs through glutamate synthase (*GLS*)-mediated conversion of cytoplasmic glutamine into glutamate. Glutamate is then metabolized in the mitochondria through *GLUD1* into alpha-ketoglutarate that enters the TCA cycle [27]. The non-canonical pathway metabolizes glutamate to aspartate and alpha-ketoglutarate through *GOT2*; aspartate is subsequently metabolized to oxaloacetate by *GOT1* in the cytosolic compartment. Aspartate is metabolized by malate dehydrogenase (*MDH*) to malate, which is then metabolized by malic enzyme (*ME*) to produce pyruvate. These anaplerotic reactions increase the NADPH/NADP ratio thereby maintaining reactive oxygen species (ROS) balance. PDAC cells are dependent on these reactions for maintenance of intracellular ROS levels as it is evidenced by the decrease in cell survival upon knockdown of enzymes in the pathway [26].

Due to metabolic reprogramming by oncogenic *KRAS* present in 90% of PDAC cases, extracellular acidification is highly abundant. While the regulation of pH in cancer cells has been studied thoroughly, the metabolic adaptations to chronic acidosis induced stress are not well defined. Therefore, in the current study, we investigated the metabolic basis of adaptation to chronic low pH stress in PDAC cells, which exhibit high glycolytic capacity, by subjecting them to chronic acidosis. We utilized PDAC cells with oncogenic KRAS to identify the metabolomic alterations under chronic acidosis and identify vulnerabilities for therapy. Here, we report a pronounced increase in non-canonical anaplerotic glutamine metabolism, which serves the bioenergetic needs and maintains ROS balance in cells undergoing acidosis stress.

Materials and methods

Cell culture

Cell culture of PDAC cell lines S2-013 and Capan-1 have been described previously [28,29]. Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich D5648) containing 4.5 g/L of glucose and 0.584 g/L of glutamine (Hyclone); additionally, the media was supplemented with 5% FBS. Low pH of the media was set at 6.9–7.1 by adding 1 g/L $NaHCO_3$ and control pH was set by using 3.7 g/L $NaHCO_3$. To establish chronic low pH exposure, we cultured the cells in pH 6.9–7.0 continuously for 14 days. Cells were maintained in low pH and control pH media for all experiments.

Cell transfections for producing replication-incompetent lentivirus were performed by utilizing Turbofect, following the manufacturer's protocol [28,30]. Stable short hairpin RNA (shRNA) constructs were obtained from Sigma-Aldrich: shGOT1 (34784; CCGGGCGTTGGTACAATGGAACAAACTCGAGTTTGTTCCATTGTACCAACGCTTTTTG) and shGOT1 (34785; CCGGGCTAATGACAATAGCCTAAATCTCGAGATTTAGGCTATTGTCATTAGCTTTTTG). Cells were transfected in control pH culture conditions and after puromycin selection and validation, scrambled controls and knockdown clones were plated in low pH for 14 days to establish chronic acidosis. Cells were validated by STR profiling.

Metabolomics

Polar metabolite isolation was performed as described previously [31]. In short, 0.75×10^7 cells were cultured for 24 h in regular DMEM. Cells were then washed with PBS and culture medium was exchanged with fresh medium 2 h before metabolite extraction, the pH of the media was maintained. Polar metabolites were then extracted with 80% methanol. Metabolite extracts were subjected to LC-MS/MS analysis using multiple reaction monitoring methods described previously [32]. Data acquisition was carried out utilizing Analyst™1.6 software (AB SCIEX), and peaks were integrated with Multiquant™ (AB SCIEX). Peak areas were normalized to the respective protein concentrations. Extraction and analysis of polar metabolites were performed three times after cells had been cultured in low pH for 14-days.

ROS assay

ROS levels were determined by using oxidation-sensitive fluorescent dye 2′,7′–dichlorofluorescin diacetate (DCFDA). Control and low pH cells were seeded at 3.0×10^4 cells per well in a clear bottom black 96-well plate. After the cells had adhered, the media was replaced with fresh DMEM containing 10 μM DCFDA, with or without respective treatments. H_2O_2 was used as a positive control and N-acetyl cysteine (NAC) was used as a negative control. Control, and treated cells were incubated at 37 °C for 30 min. The cells were washed with PBS and 100μL of PBS was added to the wells for measuring the emission of DCFDA using Biotek Cytation 3 plate reader. DCFDA was measured using an excitation of 495 nm and an emission of 529 nm. These experiments were repeated three times with similar results.

Colony formation assay

Refer to Supplemental information.

Cell cycle analysis

Refer to Supplemental information.

Glucose/glutamine uptake

Refer to Supplemental information.

Lactate release

Refer to Supplemental information.

ATP assay

Refer to Supplemental information.

Cytotoxic assays

Refer to Supplemental information.

Quantitative real-time PCR

Refer to Supplemental information.

Results

Pancreatic cancer cell growth is diminished under low pH conditions

Intracellular pH is known to have a significant role in conveying proliferation and death signals [16]. For example, it has been observed that proliferating cells require an intracellular alkaline pH value greater than 7.2, to allow growth-factor stimulated cells to enter the S-phase of the cell cycle at a faster rate, and proceed to the G_2 and M phases more rapidly [33,34]. Furthermore, a higher pH is known to suppress mitotic arrest due to activated DNA damage checkpoints; therefore, maintaining an alkaline intracellular pH enhances bypassing of cell cycle checkpoints allowing cells to have