The ketogenic diet is not effective in preclinical models of IDH1 wild-type and IDH1 mutant glioma

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ABSTRACT

Infiltrative gliomas are the most common neoplasms arising in the brain, and remain largely incurable despite decades of research. A subset of these gliomas contains mutations in *isocitrate dehydrogenase 1* (IDH1^{mut}). This mutation disrupts cellular biochemistry, and IDH1^{mut} gliomas are generally less aggressive than IDH1 wild-type (IDH1^{wt}) gliomas. Some preclinical studies and clinical trials have suggested that a ketogenic diet (KD), characterized by low-carbohydrate and high-fat content, may be beneficial in slowing glioma progression. However, not all studies have shown promising results, and to date, no study has addressed whether IDH1^{mut} gliomas might be more sensitive to KD. The aim of the current study was to compare the effects of KD in preclinical models of IDH1^{wt} versus IDH1^{mut} gliomas. *In vitro*, simulating KD by treatment with the ketone body β-hydroxybutyrate had no effect on the proliferation of patient-derived IDH1^{wt} or IDH1^{mut} glioma cells. Likewise, KD had no effect on the *in vivo* growth of these patient-derived gliomas. Furthermore, mice engrafted with Sleeping-Beauty transposase-engineered IDH1^{wt} and IDH1^{mut} glioma showed no difference in survival while on KD. These data suggest that IDH1^{mut} gliomas are not more responsive to KD, and that clinical trials further exploring KD in this subset of glioma patients may not be warranted.

INTRODUCTION

Diffusely infiltrative gliomas strike over 17,000 people in the United States per year [1]. The vast majority of these tumors recur and progress, despite advancements in surgery, chemotherapy, radiotherapy, and immunotherapy. In 20-39 year-olds, gliomas are the 2nd most common cause of cancer death in men, and are the 5th most common cause in women [1]. The most common type of primary brain cancer in adults is diffusely infiltrative glioma; the most common subtype of infiltrative glioma, glioblastoma (GBM), is unfortunately also the most lethal. Despite great advances in treating many other kinds of cancer, the median survival of GBM patients is still only 12-15 months after diagnosis, even with surgical resection, radiation, and temozolomide therapy [2, 3]. Long-term prognosis is grim; only about 15% of patients with an infiltrative glioma survive 5 years after diagnosis. As a group, primary brain cancers rank #1 among all cancers in terms of average years of life lost [4]. Despite intensive research, very little progress has been made in the treatment of GBM, and new approaches are badly needed.

Alterations in cell metabolism have long been known to be a hallmark of cancer, ever since Otto Warburg first described the preferential reliance of cancer cells on aerobic glycolysis over oxidative phosphorylation [5, 6]. However, it was only relatively recently that mutations in metabolic genes were found to occur in some cancers. For example, approximately 20-30% of infiltrative gliomas carry mutations in isocitrate dehydrogenase 1 (IDH1) or, far less commonly, IDH2 [7-9]. This subset of gliomas tends to occur in grade 2-3 gliomas, disproportionately arises in younger adults, and is associated with longer survival. Wild-type IDH1 and IDH2 encode enzymes that catalyze the oxidative decarboxylation of isocitrate to α -ketoglutarate in the cytosol/peroxisomes and mitochondrion, respectively. In the process, these enzymes also generate reduced nicotinamide adenine dinucleotide phosphate (NADPH). Point mutations in codon 132 of IDH1 (usually R132H), and codon 172 of IDH2, cause the mutant enzymes to instead reduce α -ketoglutarate to D-2-hydroxyglutarate (D2HG), thereby consuming NADPH [10].

Because cancers mostly rely on glucose for energy and anabolism, numerous studies have explored the therapeutic potential of dietary carbohydrate restriction in cancer patients. This is achieved through a prolonged ketogenic diet (KD), which is characterized by high-fat, low-carbohydrate, and adequate-protein content. KD limits the bioavailability of carbohydrates and induces the liver to produce ketone bodies, such as β -hydroxybutyrate and acetoacetate, which are then converted into acetyl-CoA for use in the tricarboxylic acid (TCA) cycle [11]. The goal of KD is to force tumor cells to use ketone bodies instead of glucose, while still meeting the patient's basic nutritional needs.

KD has been tested as an adjuvant therapeutic strategy in a number of cancers, including GBM, with mixed results thus far [12]. However, an aspect of this research that has not yet been addressed is whether patients with IDH1^{mut} gliomas might be particularly responsive to KD. One study suggested that the D2HG product of IDH1^{mut} can actually bind and inhibit ATP synthase, thereby inhibiting oxidative phosphorylation and ATP production [13]. In that study, human colorectal HCT116 cells artificially overexpressing IDH1 R132H were highly vulnerable to glucose deprivation *in vitro*, and were not able to use ketone bodies as effectively as IDH1^{wt} HCT116 cells. Therefore, we sought to explore whether KD might preferentially inhibit the growth of IDH1^{mut} gliomas *in vitro* and *in vivo*, using both patient-derived endogenous IDH1^{wt} and IDH1^{mut} xenografts in immunocompromised mice, as well as a Sleeping Beauty transposase-engineered model of IDH1^{wt} and IDH1^{mut} gliomas in immunocompetent mice.

METHODS

Ethics Statement

This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Northwestern University (protocol #5715). All surgery was performed under isoflurane inhalant anesthesia. Every effort was made to minimize animal suffering.

Cell Lines and Cell Culture

Five GBM cell types were derived from Dr. Jann Sarkaria at the Mayo Clinic Brain Tumor Patient-Derived Xenograft National Resource (<https://www.mayo.edu/research/labs/translational-neuro-oncology/mayo-clinic-brain-tumor-patient-derived-xenograft-national-resource>). Three were IDH1^{wt} (GBM6, GBM12, and GBM43), and 2 were IDH1^{mut} (GBM164 and GBM 196). Two additional IDH1^{mut} cell types were TB09, an anaplastic astrocytoma obtained from Dr Hai Yan at Duke University, and HT1080, a fibrosarcoma cell line from the American Type Culture Collection (ATCC). All IDH1^{mut} cells were R132H except for HT1080, which was R132C IDH1. NPA and NPAC1 were rodent isogenic cell lines engineered using the Sleeping Beauty transposon system, gifted courtesy of Dr. Maria Castro from the University of Michigan. Both NPA and NPAC1 have activating mutations in *NRAS* and inactivating mutations in *TP53* and *ATRX*; NPAC1 also expresses IDH1 R132H [14]. All IDH1^{mut} cell types produced high amounts of D2HG via liquid chromatography-mass spectrometry, relative to the IDH1^{wt} cells (not shown). All cell types are authenticated annually via short tandem repeat analysis.

GBM6, GBM43, TB09, and HT1080 cells were grown in Dulbecco's modified eagle medium (DMEM, Corning) supplemented with 10% fetal bovine serum and 1% penicillin streptomycin at 37°C with 5% CO₂.

In vitro proliferation

Cells were plated in 24 well plates at a concentration of 5×10^4 cells per well in triplicate. Experimental wells were supplemented with 10 mM β -hydroxybutyrate (Sigma Product #H6501). To mimic the low glucose environment characteristic of physiological ketosis, a formulation of DMEM (Corning) with 1.0 g/L of glucose was tested alongside the normal glucose concentration of 4.5 g/L. Plates were trypsinized at specific time points, and live cells were counted via trypan-blue exclusion using a BioRad TC20 Automated Cell Counter. Absolute cell counts were used to determine cell viability rather than the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, as the latter uses mitochondrial metabolism as a marker of cell viability, and we sought to avoid any potential confounding effects of culture conditions on mitochondria.

In vivo studies

GBM12, GBM164, and GBM196 cells were sourced from tumors propagated as subcutaneous growths in athymic nude mice and prepared for implantation. Briefly, after euthanizing the animal, tumors were aseptically excised from the flank and minced in a sterile culture dish with a scalpel. The cell suspension was centrifuged after mechanical disruption, filtered through 70 μ M nylon-mesh filters, re-centrifuged, and re-suspended in an equal volume ratio of cell culture media to Matrigel. A 16-gauge needle and syringe were used to inject the cell suspension into the flank of NCr female athymic nude mice (NCRNU-F sp/sp, Taconic).

Intracranial injections of NPA and NPAC1 cells were performed as described previously in 10 week-old female C57BL/6J mice (Jackson) [14].

Animals received post-operative support care through administration of 0.9% saline solution, DietGel 76A, and thermal support while recovering from anesthesia until awake and ambulatory. One administration of meloxicam 1 mg/kg was given at the time of the procedure for 24 hour analgesia coverage, followed by another dose approximately 24 hours post-procedure, and a final dose 48 hours post-procedure if deemed necessary.

126

127 *Animal monitoring and treatment*

128 All animals were fed standard rodent chow (standard diet, or SD) for 3 days following
 129 engraftment before being randomized to either remain on SD *ad libitum*, or given a formulated
 130 ketogenic diet (KD) (Ketogenic Diet TD.96355, Envigo Teklad Diets, Madison WI) *ad libitum*. The
 131 KD was received directly from the manufacturer and was a nutritionally complete diet composed
 132 of a 4.25 ratio of fat to protein plus carbohydrate (15.3% protein, 0.5% carbohydrate, 67.4% fat).
 133 Animals were placed on a weekly cyclic diet interchanging between SD and KD to control their
 134 weights and more effectively maintain plasma ketone levels, as described elsewhere [15].
 135 Animals were monitored bi-weekly for signs of morbidity such as weight loss, behavioral changes,
 136 and hunched positions, and were euthanized when tumor size approached the recommended
 137 limit of 2000 mm³, or when moribund, via CO₂ asphyxiation followed by cervical dislocation.

138 Flank tumor volume was based on caliper measurements, and calculated using the
 139 modified ellipsoidal formula [16, 17]: $V = (\text{length} \times \text{width}^2) / 2$.

140

141 *Statistical Analyses*

142 Differences between mean values of two groups were compared using two-sample *t*-test,
 143 or between multiple groups by one-way analysis of variance (ANOVA) and post hoc Tukey's test;
 144 *P* values less than 0.05 were considered significant. Log-rank tests compared survival between
 145 groups. Graph generation and statistical analyses were performed with GraphPad Prism 5
 146 (version 5.02, GraphPad Software, San Diego, CA).

RESULTS

First, we examined the effects of a ketogenic-like diet on cultured IDH1^{wt} and IDH1^{mut} patient-derived cancer cell lines under the following conditions: (i) normal basal glucose (NG) of 4.5 g/L; (ii) low glucose (LG) of 1.0 g/L; (iii) NG with 10 mM β -hydroxybutyrate (BHB); (iv) LG with BHB (**Fig 1**). Neither IDH1^{wt} GBM6 nor IDH1^{mut} HT1080 cells showed any difference in cell number across the four culture conditions over time (**Fig 1A** and **Fig 1D**). Interestingly, IDH1^{wt} GBM43 and IDH1^{mut} TB09 cells showed the greatest cell number when grown in LG +BHB (**Fig 1B** and **Fig 1C**).

Fig 1. Effect of ketones on patient-derived IDH1^{wt} and IDH1^{mut} tumor cell proliferation *in vitro*. Absolute cell counts over four days of culture in either normal glucose (NG) or low glucose (LG), with or without 10 mM β -hydroxybutyrate (BHB). **P*<0.05 by one-way ANOVA.

Next, we evaluated the ability of KD to affect the growth of patient-derived IDH1^{wt} and IDH1^{mut} GBM cells in the flanks of immunocompromised mice (**Fig 2**). (The flank was chosen because IDH1^{mut} GBM164 and GBM196 cells grow poorly as intracranial xenografts.) No significant difference in tumor volume was found in KD mice versus control among any of the tumor types.

Fig 2. The effect of KD on patient-derived IDH1^{wt} and IDH1^{mut} flank xenograft growth. Tumor volume measurements for (A) IDH1^{wt} GBM12 at 33 days post-engraftment, (B) IDH1^{mut} GBM164 at 69 days, (C) IDH1^{mut} GBM 196 at 52 days, either on normal control diet or KD. Endpoints differed due to differing rates of tumor growth.

Finally, to assess the effect of KD on orthotopic gliomas in the presence of an intact immune system, we engrafted IDH1^{wt} NPA and IDH1^{mut} NPAC1 cells into the brains of

immunocompetent mice (**Fig 3**). Among mice maintained on a regular diet, those engrafted with NPAC1 cells survived 20% longer than mice engrafted with NPA cells (median survival 26.5 days versus 22.0 days, HR=0.11, 95% CI=0.03-0.39, $P=0.0022$). However, KD had no effect on survival in either NPA-engrafted subjects (HR=0.47, 95% CI=0.15-1.5, $P=0.27$) (**Fig 3A**) or NPAC1-engrafted subjects (HR=1.0, 95% CI=0.31-3.2, $P=0.81$) (**Fig 3B**).

Fig 3. The effect of KD on patient-derived IDH1^{wt} and IDH1^{mut} intracranial xenograft growth.
Kaplan-Meier survival curves of mice engrafted with (A) IDH1^{wt} NPA or (B) IDH1^{mut} NPAC1, either on normal control diet or KD.

DISCUSSION

Altered metabolism in cancer cells raises the possibility of exploiting metabolic vulnerabilities to retard tumor growth. KD is designed to do this by depriving cancer cells of glucose, since normal cells are better able to utilize ketone bodies for their energy needs. While KD may indeed have efficacy against some cancers, clinical studies have been limited in gliomas [18]. One recurring limitation has been the lack of comparison between IDH1^{wt} and IDH1^{mut} glioma subtypes. A prior study by others suggested a potential sensitivity of IDH1^{mut} cancer to KD, based on *in vitro* data using engineered HCT116 cells [13]. Thus, we sought to determine KD efficacy in a variety of preclinical *in vitro* and *in vivo* models of IDH1^{wt} and IDH1^{mut} glioma. Our data suggest that KD does not have sufficient activity against either IDH1^{wt} or IDH1^{mut} glioma to warrant further investigation in clinical trials.

Since the original studies describing IDH1^{mut} and its neoenzymatic activity in cancer, a great deal of research has been done studying the metabolic effects of IDH1^{mut}, often generating conflicting results. For example, whereas some have shown that IDH1^{mut} depletes the cell of TCA intermediates [19-24], others have found little to no change [10, 25, 26], and that IDH1^{mut} gliomas may use lactate and glutamate anaplerosis to replenish TCA intermediates [27]. Some have suggested that glycolysis is reduced in IDH1^{mut} gliomas [21, 25]. However, one group found reduced glucose uptake by IDH1^{mut} glioma cells, but otherwise no difference in the rate of glycolysis compared to IDH1^{wt} cells [26].

Results in IDH1^{mut} metabolic research seem to vary greatly depending on whether one is studying cells overexpressing IDH1^{mut} via transduction or is focusing on cells and tissues with endogenous, IDH1^{mut}, as more pronounced metabolic changes tend to occur in the former than the latter [26]. This suggests that cells with endogenous IDH1^{mut} can, over time, adjust their metabolism to at least partially compensate for perturbations caused by the mutant enzyme. For example, IDH1^{mut} gliomas may compensate for the depletion of TCA intermediates by upregulating glutamate dehydrogenase 2 expression [21]. Since IDH1^{mut} gliomas mostly use the

TCA precursor glutamine to produce D2HG, these tumors compensate by turning pyruvate into TCA intermediates [28, 29]. While IDH1^{mut} consumes NADPH, which should lead to glutathione depletion, IDH1^{mut} gliomas upregulate enzymes involved in glutathione synthesis, thereby maintaining glutathione levels [30]. Thus, when IDH1^{wt} wild-type cells are abruptly forced to overexpress IDH1^{mut}, any metabolic results, including sensitivity to KD-like conditions, need to be validated in patient-derived and/or transgenic models with endogenous IDH1^{mut}.

Prolonged adherence to KD is notoriously difficult [31]. For a large well-controlled KD clinical trial to be justified in glioma patients, the preclinical evidence would therefore need to be particularly compelling. Our results suggest that KD is not a promising therapeutic strategy in glioma patients, regardless of IDH1^{mut} status.

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Figure 1

A

IDH1^{wt} NPA
HR=0.47 (95% CI=0.15-1.5)
P=0.27

B

IDH1^{mut} NPAC1
HR=1.0 (95% CI=0.31-3.2)
P=0.81

Figure 3

Figure 2