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Glucose Metabolism via the Pentose Phosphate Pathway, Glycolysis and Krebs Cycle in an Orthotopic Mouse Model of Human Brain Tumors

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Abstract

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It has been hypothesized that increased flux through the pentose phosphate pathway (PPP) is required to support the metabolic demands of rapid malignant cell growth. Using an orthotopic mouse model of primary human glioblastoma (GBM) and a brain metastatic renal tumor of clear cell renal cell carcinoma (CCRCC) histology, we estimated the activity of the PPP relative to glycolysis by infusing [1,2- $^{13}\text{C}_2$]glucose. The [3- ^{13}C]lactate/[2,3- $^{13}\text{C}_2$]lactate ratio was similar for both the GBM and renal tumor and their respective surrounding brains (GBM: 0.197 ± 0.011 and 0.195 ± 0.033 ($p=1$); CCRCC: 0.126 and 0.119 ± 0.033 , respectively). This suggests that the rate of glycolysis is significantly greater than PPP flux in these tumors, and that PPP flux into the lactate pool was similar in both tissues. Remarkably, ^{13}C - ^{13}C coupling was observed in molecules derived from Krebs cycle intermediates in both tumors, denoting glucose oxidation. In the renal tumor, in contrast to GBM and surrounding brain, ^{13}C multiplets of GABA differed from its precursor glutamate, suggesting that GABA did not derive from a common glutamate precursor pool. Additionally, the orthotopic renal tumor, the patient's primary renal mass and brain metastasis were all strongly immunopositive for the 67-kDa isoform of glutamate decarboxylase, as were 84% of tumors on a CCRCC tissue microarray suggesting that GABA synthesis is cell-autonomous in at least a subset of renal tumors. Taken together, these data demonstrate that ^{13}C -labeled glucose can be used in orthotopic mouse models to study tumor metabolism *in vivo* and to ascertain new metabolic targets for cancer diagnosis and therapy.

Keywords

Intermediary metabolism; Mouse model; ^{13}C NMR; brain; glioblastoma; renal cell carcinoma

INTRODUCTION

The pentose phosphate pathway (PPP) provides ribose 5-phosphate for nucleotide synthesis and NADPH as a cofactor for fatty acid synthesis. These metabolites are required for cell proliferation and for this reason flux in the PPP is thought to be increased in cancer [1, 2]. This hypothesis is consistent with high uptake of ^{18}F fluoro-deoxyglucose in tumors detected by positron emission tomography (^{18}F FDG-PET). However, flux in the PPP relative to glycolysis is difficult to examine in cancer *in vivo* because of the complex connections between the two pathways. Methods to monitor the PPP take advantage of the first few biochemical steps. The oxidative portion of the pathway involves an initial reduction of glucose-6-phosphate to form 6-phosphogluconolactone plus NADPH, followed by conversion to 6-phosphogluconate which in turn undergoes decarboxylation at position 1 to yield CO_2 , a second molecule of NADPH, and ribose-5-phosphate (Figure 1). All carbon tracer methods probing the pathway rely on detecting the effects of this characteristic decarboxylation reaction in downstream metabolites, particularly lactate. Traditionally ^{14}C -enriched glucose was used in rodent and human experiments [3-5] but more recent studies have extensively evaluated the use of [2- ^{13}C]glucose or [1,2- $^{13}\text{C}_2$]glucose with detection of lactate by either ^{13}C NMR spectroscopy [6-9] or mass spectrometry [10]. Together, these studies indicate that flux of glucose through the PPP is active in a variety of tissues and that it is increased in cancer [11] or after brain injury [12].

Although established tumor cell lines have provided invaluable insights into the regulation of metabolic pathways, recent molecular studies have shown that, *in-vitro*, especially under serum enriched cell culture conditions, tumor cells may undergo marked genomic and epigenetic changes [13, 14]. How cell culture conditions may modify tumor cell metabolism or the extent to which these artificial conditions are able to replicate the *in situ* tumor environment is unknown. To minimize non-physiological metabolic changes in the cell, we developed an orthotopic mouse model of human brain tumors that provides a good approximation of the cells' native microenvironment. Using this model, we analyzed the

relative activity of the PPP compared to glycolysis in orthotopic glioblastoma and orthotopic clear cell renal cell carcinoma (CCRCC) brain metastasis *in situ* by infusion of [1,2-¹³C₂]glucose followed by microdissection and analysis of the tissue extract by ¹³C NMR spectroscopy. The distribution of ¹³C in lactate was used to assess flux through the PPP relative to glycolysis essentially as described by others[10, 12, 15].

Flux through the PPP relative to glycolysis was not significantly different between the GBM and CCRCC and their respective nontumor-bearing surrounding brains. As anticipated, glucose was oxidized *via* pyruvate dehydrogenase and Krebs cycle in non tumor-bearing surrounding brain tissue with label observed in glutamine as well as in the neurotransmitters, glutamate and GABA. Surprisingly, oxidation of glucose in the Krebs cycle was observed in both GBM and a CCRCC. Because GABA was detected in the renal tumor and CCRCC brain metastasis and GABA synthesis is not characteristic of normal renal metabolism, additional immunohistochemistry studies were performed to assess the distribution of glutamic acid decarboxylase, the key enzyme in GABA synthesis, in the CCRCC brain metastasis. Taken together, our data show that an orthotopic mouse brain tumor model can be used to assess intermediary metabolism in the context of normal brain microenvironment. This approach may enable detection of novel biomarkers of specific tumors.

Experimental

Orthotopic Transplantation of Human Tumors into Mouse Brain

Brain tumor tissue was collected from three patients with a diagnosis of primary glioblastoma (GBM) and one patient with a metastatic renal tumor of clear cell renal cell carcinoma (CCRCC) histology, undergoing complete surgical resection at the University of Texas Southwestern Medical Center. Written informed consent for use of tumor tissue was obtained from all patients under a protocol approved by the Institutional Review Board. Diagnoses were established by pathological examination. Tumors were collected in ice-cold Hanks medium and were gently dissociated by enzymatic treatment with papain. A single cell suspension ($\sim 1 \times 10^5$) was injected into the right caudate of 6-week-old female NOD/SCID mice as previously described[16]. A cohort of 5 mice were injected for each of the 3 GBM tumors and one renal cell tumor (n=20 mice for this study). All tumor cell transplantations were completed within 3 hours of surgical resection. Mice were examined twice weekly for weight loss, loss of grooming, seizures, and focal motor deficits.

Magnetic Resonance Imaging and Positron Emission Tomography (PET)

Brain imaging was performed to document the presence of an expanding intracranial mass. MR imaging was conducted with a 7-T small animal MR system (Varian, Inc, Palo Alto, CA) with a 40 mm (I.D.) Horizontal Millipede™ Coil. All animals were anesthetized with 1-2% isoflurane (AERRANE, Baxter Healthcare Corporation, IL) mixed in 100% oxygen and placed head first in the supine position with a respiratory sensor. The head was centered with respect to the center of the RF coil. First, low-resolution multi-slice imaging was performed on the head region to confirm the location and orientation of the brain. Precontrast T1-weighted (repetition time/echo time, 500/10 msec; echo-train length, 2; field of view, 25.6×25.6 mm; matrix, 256×256; in-plane resolution, 100×100 μm; slice thickness, 1 mm; gapless; number of excitations, 4; number of slices, 11) and T2-weighted (repetition time/echo time, 2500/60 msec; echo-train length, 8; field of view, 25.6×25.6 mm; matrix, 256×256; in-plane resolution, 100×100 μm; slice thickness, 1 mm; gapless; number of excitations, 4; number of slices, 11) multi-slice images were obtained with a fast spin-echo sequence in the axial plane. Following an i.v. injection of 0.2 mmol/kg gadolinium contrast agent (Prohance, Bracco Diagnostic Inc, Princeton, NJ), postcontrast T₁-weighted images were acquired with identical parameters as above. Total acquisition time for each animal

was approximately 20 minutes. The mice were monitored for respiration during the imaging session with an MR imaging-compatible small-animal respiratory gating device (SA Instruments, Inc., Stony Brook, NY).

^{18}F FDG-PET imaging was performed in a small animal PET scanner with a full-width at half-maximum resolution of 2 mm was used. GBM-bearing mice were anesthetized with 2% isoflurane. Following positioning in the scanner, an attenuation data set was obtained; then animals were injected with 35 mCi of ^{18}F FDG through the tail vein and maintained in the scanner for 1 h for real-time acquisitions to ensure the generation of regions of interest (ROI) (tumor vs normal contralateral brain) from which time-activity curves were constructed.

Intravenous Infusion of $[1,2-^{13}\text{C}_2]\text{glucose}$

Tracer studies were performed when mice developed focal neurological deficits. This typically occurred at 4-6 weeks for NOD-SCID mice implanted with the CCRCC brain metastasis and at 10-12 weeks for GBM orthotopic tumors. The right jugular vein was aseptically cannulated under 2% isoflurane. The catheters were filled with glycerol to maintain patency. After cannulation, animals were allowed to recover from anesthesia and were loosely confined to a cylindrical Lucite cage. $[1,2-^{13}\text{C}_2]\text{glucose}$ (99% enrichment; Cambridge Isotope Laboratories, Cambridge, MA) was administered as a bolus of 0.4mg/g (0.3 ml) infused in ~1 min, followed by a continuous infusion of 0.012 mg/g/min at 150 μl /hr for a duration of 150 min. At the end of the infusion, blood was collected by retro-orbital puncture (100-150 μl). The mice were then deeply anesthetized and decapitated, the brain was rapidly removed from skull (<30sec), immersed into ice-cold Hanks media and cut into 1mm coronal sections on pre-chilled rodent brain block and examined by trans-illumination under a dissecting microscope. Orthotopic CCRCC tumors were easily identified by a distinct border between tumor mass and surrounding normal brain. GBM orthotopic tumors were characterized by a large central mass surrounded by indistinct borders at the tumor margins. The central tumor mass and the contralateral hemisphere were separately microdissected and weighed (GBM: 217 ± 28 mg, renal cell: 44 ± 7 mg, surrounding brain of GBM: 376 ± 53 g, surrounding brain of renal cell: 361 ± 50 g), frozen in liquid nitrogen, and stored at -85°C . ~20 μL of blood was saved to measure blood glucose concentration after the infusion with a Contour® glucometer (Bayer Healthcare, Tarrytown, NY)[17] and/or an enzyme spectrophotometric assay (GAHK20-1KT kit, Sigma, St Louis, MO). The same tumor extraction protocol was carried out for the sham-injected NOD-SCID mouse brain.

Preparation of Tumor Extracts

The mass of CCRCCs ranged from 30-50 mg per tumor at dissection. In preliminary experiments we had established that 150 mg (wet weight) of tissue was required to generate a reasonable NMR spectrum (data not shown). Therefore, to have an adequate sample for NMR analysis, we pooled 4 orthotopic CCRCCs. Pooled tumors and pooled non-tumor bearing surrounding brain from each mouse were finely ground in a mortar under liquid nitrogen. Perchloric acid (4%; 1:4 w/v) was added to each sample, followed by centrifugation at 47800 g for 15 min. The supernatant was transferred to a new tube where chloroform/tri- n -octylamine (78%/22%; v/v) was added in a 1:2 volumetric ratio to increase the pH to ~6. The samples were centrifuged at 3300 g for 15 min, the aqueous phase removed and transferred to a microfuge tube and then lyophilized. 100 μl of deuterium oxide (99.96%, Cambridge Isotope Laboratories) was added to each sample and the pH adjusted to 7.0 with 2-3 μL of 1 M of sodium deuterioxide (99.5%, Cambridge Isotope Laboratories). The pH-neutral samples were then centrifuged at 18,400 g for 1 min and the supernatant removed and placed into a 3-mm NMR tube for subsequent NMR analysis.

Measurement of ^{13}C Fractional Enrichment in Blood Glucose

Blood samples were processed to measure ^{13}C -glucose enrichment by gas chromatography-mass spectrometry (GC-MS). A 10 μL aliquot of whole blood was transferred to a 16 $\times$ 10mm glass tube. Glucose was extracted by the sequential addition and vortexing of 2 mL each of methanol, chloroform and water. Samples were then centrifuged at 2000g for 5 min. Approximately 3.5 mL of the upper aqueous phase was transferred to a new glass tube and extracted with 2ml chloroform. After centrifugation at 2000 g for 5 min, approximately 3 ml of the upper aqueous phase was transferred to a screw-topped glass tube, and evaporated to dryness under blown air at 42 °C. The samples were then derivatized by adding 100 μL Tri-Sil reagent (Pierce, Thermo Fisher Scientific, Rockford, IL), vortexing, capping, and heating for 30 min at 42° C. A glass Pasteur pipette was used to transfer each sample into an autoinjector vial for GC-MS. A three-point standard curve was also prepared using mixtures of un-enriched glucose with [1,2- $^{13}\text{C}_2$]-glucose such that 0, 50 or 100% of the glucose in the final mixture was ^{13}C -labeled. GC-MS was performed using an Agilent 6890N Gas Chromatograph coupled to an Agilent 5973 Mass Selective Detector (Agilent Technologies, Santa Clara, CA). One microliter of each standard or sample was injected and analyzed in scan mode. Abundances of fragment ions 204 (unenriched) and 205 (enriched) were determined for each standard and sample, and the ratio of 205/(204+205) ions was calculated. A standard curve was generated and linear regression was used to calculate the atom percent excess (APE) of glucose from the 205/(204+205) ratio for each sample.

^{13}C NMR Spectroscopy

Proton decoupled ^{13}C spectra were acquired on a 400 MHz Varian magnet and Varian VNMRs Direct Drive console using a 5 mm auto-switchable broadband probe (Varian Instruments, Palo Alto, CA). Proton decoupling was performed using a Waltz-16 sequence. ^{13}C NMR parameters include a 45 degree flip angle per transient, a relaxation delay of 1.5 sec, an acquisition time of 1.5 sec, and spectral width of 24.5 kHz. Samples were spun at 20 Hz with the temperature regulated at an ambient 25 °C. A ^2H field-frequency lock was used.

Analysis of Spectra

NMR spectral analyses were performed with ACD/Spec Manager 11.0 software (Advanced Chemistry Development, Inc., Toronto ON, Canada). Free induction decays were zero-filled and multiplied by an exponential weighting function of 0.5 to 1.0 Hz prior to Fourier transformation. Resonances were assigned based on chemical shift position referenced to the glutamate C4 singlet at 34.2 ppm. Each signal was fitted with a Gauss-Lorentz function and the area measurements for each fitted resonance peak and the multiplet areas estimated as previously described[18, 19].

^{13}C NMR isotopomer analysis of glutamate measures the labeling pattern of acetyl-CoA feeding the Krebs cycle. The analysis of glutamine is equivalent to glutamate. Since GABA is produced from glutamate, the ^{13}C NMR spectrum of GABA also provides information about acetyl-CoA labeling patterns. Specifically, the ^{13}C labeling patterns in glutamate carbons 2–5 are preserved in GABA except that the designation of labeled carbons is reversed (carbon 1 in GABA corresponds to carbon 5 in glutamate, etc., see Supplementary Figure 2). Because of the structure of the Krebs cycle and numbering conventions, this means that the ^{13}C enrichment in C1 of GABA is equal to the ^{13}C enrichment in C1 of acetyl-CoA and the ^{13}C enrichment in C2 of GABA is equal to the ^{13}C enrichment in C2 of acetyl-CoA. The only oxaloacetate carbon positions that are detected in GABA are carbon 2 of oxaloacetate, equivalent to carbon 3 of GABA, and carbon 3 of oxaloacetate, equivalent to carbon 4 of GABA. Since the ^{13}C labeling patterns in glutamate carbons 4 and 5 encode the labeling pattern in acetyl-CoA, it is a simple matter to examine substrate competition in

GABAergic neurons, even under non-steady state conditions, using the principles described for glutamate [18, 19].

Immunohistochemistry

Paraffin-embedded GBM and renal cell carcinoma specimens were obtained from samples maintained in the Divisions of Neuropathology and Surgical Pathology at UT Southwestern Medical Center. Tissue microarray constructs of 96 renal cell carcinoma specimens were obtained from the Division of Surgical Pathology at UT Southwestern Medical Center. The GBM tissue and human orthotopic tumor-bearing mouse brains were fixed in 10% formalin and embedded in paraffin. Sections were cut at 4- μ m thickness and deparaffinized. The sections were incubated in 3% H₂O₂ for 10 minutes to block endogenous peroxidase activity. The primary antibodies and their dilutions were as follows: MIB-1 (prediluted; 790-4286 Ventana Medical Systems, Tucson, AZ) and GAD67 (1:500; ab26116; Abcam, Cambridge, MA), respectively. All immunostains were performed on a Benchmark XT stainer using the CC1 pretreatment solution (95 °C for 30 min) and XT UltraView Universal DAB detection system (Ventana). The sections were counterstained with hematoxylin.

Statistical analysis

The statistical analysis was performed with the non-parametric Wilcoxon two-sample test using SPSS Graduate Pack version 18.0 (SPSS; Chicago, IL, USA).

RESULTS

All three orthotopic primary GBM tumor lines appeared hyperintense on T2 sequences, hypointense on T1 with gadolinium contrast enhancement and showed avid ¹⁸F-DG uptake by ¹⁸F-DG-PET imaging. The mouse MR and PET imaging data were similar to the imaging data obtained pre-operatively in the GBM patients from whom the tumors lines were derived (Figure 2A). These data suggest that the routine clinical imaging modalities used to assess and follow GBM patients are reproduced in the orthotopic setting of NOD-SCID mouse brain.

Histological analysis of orthotopic intracranial GBM tumors (n=3), were characterized by a high mitotic index (Ki-67 50-70%), pleiomorphic nuclei, tortuous microvasculature and diffuse single cell infiltration. All of these features are characteristic of high grade gliomas (Figure 2B). The orthotopic CCRCCs derived directly from a CCRCC brain metastasis showed brisk proliferation (Ki-67>50%), monomorphic nuclei, scant cytoplasm and distinct margins between the tumor mass and the brain parenchyma (Figure 2B); all features consistent with the histological analysis of the resected brain metastasis from the patient. Tumor cell-host interactions defined by diffuse single cell infiltration or a distinct tumor-brain parenchyma interface are characteristic histological features of GBM and metastatic brain tumors, respectively. These were observed in the orthotopic setting of the NOD-SCID mouse brain.

Evaluation of Pentose Phosphate Pathway Activity *In Vivo*

Intravenous infusion of [1,2-¹³C₂]glucose followed by ¹³C NMR analysis of microdissected tumor mass and nontumor bearing surrounding brain, was used to assess flux of the PPP relative to glycolysis. Mean blood glucose levels at the end of the infusions were 2.8 mM (n=3, range: 1.2 to 5.7 mM) for GBM and 6.6 mM (n=4, range: 5.6 to 11.8 mM) for CCRCCs. The enrichment of ¹³C-glucose in blood was 21.3 $\pm$ 11.6 % and 55.3 $\pm$ 17 % in mice with GBM and CCRCC, respectively. The higher mean blood glucose levels and greater fractional ¹³C-glucose enrichment in the CCRCC brain metastasis cohort suggests less robust glycemic control. Whether this is the result of a rapidly growing, non-infiltrating

tumor mass, which adversely affects hypothalamic-pituitary axis, is not clear. The ^1H -decoupled ^{13}C NMR spectrum from the brain of a control NOD/SCID mouse infused with $[1,2-^{13}\text{C}_2]\text{glucose}$ is shown in Figure 3. Since glucose is the preferred substrate for energy production in normal brain, the appearance of spin-coupled ^{13}C multiplets in metabolites exchanging with the Krebs cycle such as glutamate, glutamine, GABA and aspartate is anticipated [20]. The presence of spin-spin coupling in the NMR spectrum of these metabolites confirms their origin from infused $[1,2-^{13}\text{C}_2]\text{glucose}$. The natural abundance ^{13}C signal from taurine was also detected routinely in the normal brain. The majority of the ^{13}C -enriched lactate (inset) originated from $[1,2-^{13}\text{C}_2]\text{glucose}$ via glycolysis, indicated by the high doublet D23 relative to the singlet (singlet to doublet ratio 0.69).

Blood enrichment of ^{13}C -lactate (from M+0 to M+3) was measured in GBM and CCRCC animals (Supplementary Figure 3). Most of the lactate was unlabeled (M+0) or labeled in two positions (M+2). However, the enrichment of lactate M+1, which, by definition, includes lactate labeled in C1, C2 or C3, was <1% in all animals. This indicates that the contribution of lactate M+1 to the singlets of lactate C3 and glutamate C4 was very low.

^{13}C -NMR spectra from the brain of a non-tumor-bearing mouse and from the uninvolved contralateral hemisphere from mice bearing GBM or renal cell tumor are shown in Figure 4. The singlet-to-doublet ratio of lactate C3 was not significantly different between the GBM and its surrounding brain, 0.197 ± 0.011 vs. 0.195 ± 0.033 ($p=1$), respectively, or between the CCRCC brain metastasis and its surrounding brain, 0.126 and 0.119 ± 0.033 , respectively. The relative increase in the Lactate C3 ratio in the surrounding brain of GBM when compared to brain surrounding the CCRCC likely reflects the relative decrease in brain perfusion with a large tumor mass (217 mg, GBM vs 44 mg for CCRCC). This data, in addition to the presence of the same pattern in non-tumor bearing brain (large doublet relative to the singlet) suggests that flux through the pentose phosphate pathway relative to glycolysis is not increased in the tumors compared to the surrounding non-tumor bearing brain.

Spectra from mice with an intracranial tumor showed multiplets in glutamate carbon 4 that were dominated by the doublet resulting from coupling between carbon 4 and 5 (D45) (Figure 4). This doublet was derived from $[1,2-^{13}\text{C}_2]\text{acetyl-CoA}$. The quartet (doublet of doublets) resulting from glutamate labeled in positions 3 and 4 and 5 appears as a consequence of turnover of the Krebs cycle. The singlet in glutamate C4 is due to natural abundance ^{13}C or to more complex pathways such as pyruvate recycling [21], which produces $[2-^{13}\text{C}]\text{acetyl-CoA}$. Importantly, there was no qualitative difference in the ^{13}C spectrum of GABA C2 (at 35.1 ppm) compared to glutamate C4 (at 34.2 ppm). If most or all GABA is produced directly from brain glutamate, these multiplets should be identical.

Production of Glutamate and GABA in Orthotopic Tumors

In both GBM and CCRCC brain metastasis, ^{13}C multiplets were also observed in Krebs cycle -derived metabolites, such as glutamate and GABA. In GBM, GABA C2 had essentially the same labeling pattern as glutamate C4 (Figure 5A). These highly similar multiplets are expected for a direct precursor-product relationship, as predicted for GABA, which is produced from glutamate by a single decarboxylation catalyzed by glutamate decarboxylase (e.g. GAD67, glutamate decarboxylase). Extensive brain neurochemical analysis has established that GAD67 is exclusively expressed in a population of cortical and subcortical interneurons, so called GABAergic neurons that convert glutamate to GABA[22]. This hypothesis is supported by immunohistochemical (IHC) evidence showing inclusion of GABAergic interneurons interspersed within the infiltrating GBM tumor mass (Figure 5A, blue arrows).

In contrast, the CCRCC brain metastasis, which is devoid of infiltrating normal brain parenchyma (except, host derived endothelial cells), exhibited a higher doublet, D12, relative to singlet, S, in GABA C2 compared to doublet, D45, to singlet, S, in glutamate C4, resulting in a singlet-to-doublet ratio of 0.172 and 0.346, respectively (Figure 5B). Although this observation was obtained from a single spectrum (produced by pooling CCRCC tissue from the brains of 4 mice), these data indicate that GABA production in the CCRCC brain metastasis originates from a subset of cells that do not produce the majority of the glutamate.

GAD67 Expression in Human Clear Cell Renal Cell Carcinomas

To further explore the unexpected finding of GABA synthesis in the CCRCC brain metastasis, we examined the orthotopic tumor tissue for expression of the 65 kDa and 67 kDa isoforms of GAD (GAD65 and GAD67), respectively, by immunohistochemistry. The CCRCC brain metastasis tumors were strongly immunopositive for GAD67 (Figure 5B), but not for GAD65 (data not shown). Furthermore, immunohistochemistry evaluation of the human brain metastatic tumors from which the orthotopic tumors were derived revealed extensive GAD67 staining (Figure 6). In contrast, GAD67 staining was confined to the interneurons in the GBM (Figure 5A), consistent with the lack of expression of GAD67 in GBM cells. As further strong evidence for GABA production by the CCRCC brain metastasis, we obtained tissue sections from the patient's primary kidney mass, and demonstrated that it also stained positively for GAD67 (Figure 6). Finally, to test whether GAD67 expression is a common feature of renal tumors with 'clear cell renal cell carcinoma' histology, we stained a tissue microarray of an additional set of 96 primary human renal tumors with the same histological (CCRCC) diagnosis (supplementary Figure 1). Remarkably, 84% stained for GAD67. Seventeen (17.7%) demonstrated strong immunoreactivity (3+/3), 34 (35.4%) demonstrated moderate (2+/3) immunoreactivity, 30 (31.3%) were mildly immunoreactive and only 15 (15.6%) showed no immunoreactivity to GAD67 (0/3). The staining pattern showed no statistically significant correlation with tumor grade, tumor stage, presence or absence of metastasis (data not shown). Normal human kidney epithelium, the presumptive origin of clear cell carcinomas, showed no GAD67 immunoreactivity (data not shown).

DISCUSSION

^{13}C -labeling in lactate during infusion of $[1,2-^{13}\text{C}_2]\text{glucose}$ was used to assess the relative activity of the PPP compared to glycolysis in orthotopic mouse models of GBM and CCRCC that had metastasized to the brain. There was no significant difference in the singlet/doublet ratio in carbon 3 of lactate between either the GBM or CCRCC and surrounding non-tumor bearing brain tissue. Interestingly, all tumors showed excess uptake of ^{18}F FDG on PET scan. Taken together and assuming that increased ^{18}F FDG signal indicates increased glucose metabolism, these results indicate that the relative flux through the PPP compared to glycolysis is not altered in these tumors but the combined rate of glucose metabolism is increased. This estimation of the PPP relative to glycolysis was based on the singlet-to-doublet ratio of lactate C3 derived from $[1,2-^{13}\text{C}_2]\text{glucose}$. This approach assumes that the singlet arising from lactate C3 is the product of the oxidative branch of the PPP plus the background natural abundance signal (1.1% of total carbon). In both the GBM and CCRCC brain metastasis, the ratio of singlet to doublet in lactate C3 was similar between the tumor and the surrounding brain. Although the current data could be interpreted as excess flux through the PPP in both tumor and surrounding brain relative to normal brain, it is not possible to draw that conclusion with confidence because of possible compartmentation of lactate. Specifically, lactate in the tumor mass is present in at least three potential compartments: in blood, in a slowly-exchanging pool in poorly perfused or

metabolically inactive tumor cells, and in a rapidly-exchanging pool that reflects metabolism of exogenous glucose. We have shown that the majority of lactate in blood is not enriched, thus metabolism of lactate from blood does not contribute to the PPP. Alternatively, PPP flux can be underestimated using this approach, as ribose 5-phosphate generated through the oxidative branch of PPP may not reenter glycolysis. Instead, it can be used for nucleotide synthesis, reducing the abundance of lactate C3 singlet.

Previous studies had suggested that while the bioenergetic demands of malignant cell growth are met by enhanced glycolysis[1, 2], the PPP is preferentially activated to support macromolecule biosynthesis that is necessary for cellular growth[23, 24]. It is of interest to note, however, that estimates of maximum flux through PPP relative to glycolysis reported here for GBM and CCRCC tumors (19% for and 12%, respectively) are comparable to those reported in traumatic brain injury in a rodent model (9-12%) and humans (19.6%)[12, 15]. These estimates are significantly higher than the approximate 5% estimates of PPP activity in brain under unstressed conditions [25, 26]. One possible explanation for why PPP activity may be increased by TBI or by the presence of a large intracranial mass and TBI tissue is that both circumstances are associated with vasogenic/cytotoxic edema. This could produce a compressive force as the brain expands inside a rigid cranium compromising perfusion and limiting oxygen diffusion as result of interstitial edema. Although speculative, it is possible that the resulting oxidative stress (generation of ROS) may explain why both intracranial tumor tissue and non-tumor bearing regions of the brain show increased PPP activity.

^{13}C NMR analysis of both GBM and CCRCC brain metastasis, revealed ^{13}C - ^{13}C coupling in glutamate and GABA, suggesting that glucose was metabolized to acetyl-CoA and further oxidized in the Krebs cycle. Although $[1,2\text{-}^{13}\text{C}_2]$ labeled glucose is a suboptimal tracer for assessing Krebs cycle intermediates since only 50% of acetyl-CoA becomes ^{13}C labeled, nevertheless the presence of multiplets in glutamate and GABA denotes presence of Krebs cycle activity. This observation suggests that malignant tumors growing in the microenvironment of the brain rely on oxidative metabolism in addition to glycolysis and increased production of lactate, typical of the Warburg effect[2].

An unexpected observation in the CCRCC brain metastasis was that ^{13}C multiplets in GABA C2 displayed a lower singlet-to-doublet ratio (0.172) relative to its precursor glutamate C4 (0.346), suggesting that GABA derived from a subset of the total glutamate pool. This was not observed in spectra from normal brain or GBM. Conversion of glutamate to GABA requires GAD67 (or GAD65) which, in the brain, is exclusively expressed by a critically important subset of GABAergic inhibitory interneurons [22]. Although the orthotopic metabolic data presented here was derived from a single CCRCC brain metastatic tumor, further support for this novel finding comes from the demonstration that the patient's brain metastasis, from which the orthotopic tumor line was derived, as well as the patient's primary renal tumor mass, all showed strong GAD67 immunoreactivity. Moreover, the importance of this discovery and its potential clinical relevance is demonstrated by the finding that 86% of an independently generated, clinically annotated, tissue microarray of 96 individual renal tumors with CCRCC histopathology also showed GAD67 immunoreactivity. Since the expression level of GAD67 did not appear to correlate with tumor grade or prognosis, it raises the possibility that upregulation of GAD67 expression is an early event in the genesis of CCRCC tumors. The fact that elevated GAD67 expression persists as CCRCCs accrue mutations and evolve into higher grade local and metastatic disease, including brain, suggests that the ability to synthesize GABA may play an important role in the pathophysiology of this disease. In support of the speculation that GABA signaling may promote tumor growth, there is growing evidence that a wide variety of non-CNS tumors express GAD67, synthesize GABA and express GABA-specific receptors

(GABA-A and GABA-B) [27]. What specific role GABA plays in supporting renal tumor growth, particularly brain metastasis remains to be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

¹³C	carbon 13
¹⁸FDG-PET	¹⁸ fluoro-deoxyglucose positron emission tomography
6PG	6-phosphogluconate
6PGDH	6-phosphogluconate dehydrogenase
APE	atom percent excess
ASP	aspartate
CCRCC	clear cell renal cell carcinoma
CO₂	carbon dioxide
D23	doublet 23
F6P	fructose 6-phosphate
G3P	glyceraldehyde 3-phosphate
G-6PDH	glucose 6-phosphate dehydrogenase
GABA	<i>gamma</i> -Aminobutyric acid
GAD	glutamate decarboxylase
GBM	glioblastoma
GC-MS	gas chromatography-mass spectrometry
Glc	glucose
Glc-6-P	glucose 6-phosphate
GLN	glutamine
GLU	glutamate
HK	hexokinase
LAC	lactate
LDH	lactate dehydrogenase
MIB-1	monoclonal antibody to Ki67
MR	magnetic resonance
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance

NOD/SCID	non-obese diabetic/severed combined immunodeficient
PET	positron emission tomography
PGI	phosphoglucose isomerase
PPP	pentose phosphate pathway
PYR	pyruvate
R5P	ribose 5-phosphate
S3	singlet 3
TAU	taurine

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FIGURE 1.

Diagram depicting [1,2-¹³C₂]-glucose metabolism through glycolysis and the pentose phosphate pathway (PPP) with the resulted labeling pattern of lactate C3. As a consequence of the higher activity of glycolysis relative to PPP in normal cells, the contribution of the doublet (D23) to the total NMR signal of lactate C3 is significantly more prominent than the singlet (S3). HK: hexokinase, G-6PDH: glucose 6-phosphate dehydrogenase, 6PGDH: 6-phosphogluconate dehydrogenase, PGI: phosphoglucose isomerase, LDH: lactate dehydrogenase, Glc: glucose, Glc-6-P: glucose 6-phosphate, F6P: fructose 6-phosphate, G3P: glyceraldehyde 3-phosphate, 6PG: 6-phosphogluconate, R5P: ribose 5-phosphate, PYR: pyruvate, LAC: lactate. Open circles denote carbon 12, filled circles denote carbon 13.

FIGURE 2.

Orthotopic primary GBM mouse tumors share MR and PET imaging features seen in original patient. In panel A, both human and orthotopic mouse GBM tumors appear as a large hyperintense T2 mass, with significant mass effect (mouse MRI). Clinical and orthotopic tumor FDG-PET images are consistent with enhanced glucose uptake relative to the surrounding brain tissue. Panel B shows hematoxylin-eosin (H&E) and Ki67 staining from the orthotopic GBM and orthotopic CCRCC brain metastasis. GBM tumors were characterized by a high mitotic index, pleomorphic nuclei, tortuous microvasculature and diffuse single cell infiltration. In contrast, the CCRCC showed brisk proliferation, monomorphic nuclei and scant cytoplasm. Magnification of histological images is 20 $\times$.

FIGURE 3.

Section of the forebrain NMR spectrum from a non-tumor bearing NOD/SCID mouse infused with [1,2- ^{13}C]-glucose. Spin-coupled ^{13}C multiplets in metabolites exchanging with Krebs cycle intermediates (i.e., glutamate, glutamine, GABA and aspartate) is observed in the ^{13}C spectrum as a consequence of glucose oxidation in the brain. Of note, the labeling pattern of glutamate C4 (GLU4) and GABA C2 (GABA2) were comparable as GABA derives primarily from glutamate in normal brain. Insets display labeling patterns of lactate C3 and from aspartate C3 to glutamine C4. GLU: glutamate, GLN: glutamine, ASP: aspartate, LAC: lactate, TAU: taurine. 1: N-acetylaspartate C6, 2: GABA C3, 3: unassigned, 4: glutamine C3, 5: glutamate C3, 6: unassigned, 7: unassigned, 8: GABA C4, 9: N-acetylaspartate C3, 10: taurine C1, 11: aspartate C2, 12: N-acetylaspartate C2, 13: unassigned, 14: glutamine C2, 15: glutamate C2. S: singlet, Dxx: doublet, Q: quartet.

FIGURE 4. Section of the ^{13}C -NMR spectrum illustrating the labeling pattern of glutamate C4 (GLU4), GABA C2 (GABA2), and Lactate C3 (LAC33) in normal forebrain (A), surrounding brain of GBM (B), surrounding brain of CCRCC metastatic to the brain (C). The labeling pattern between both isotopomers is notably consistent throughout all brain tissues. S: singlet, Dxx: doublet, Q: quartet.

FIGURE 5.

Section of the ¹³C-NMR spectrum showing the labeling pattern of glutamate C4 (GLU4) and GABA C2 (GABA2) in GBM (A) and CCRCC orthotopic tumors (B) and their respective immunohistochemical staining for GAD 67 (20×). In contrast to GBM, in the CCRCC orthotopic tumor GABA2 showed a lower singlet-to-doublet ratio relative to its precursor GLU4, consistent with compartmentalization of GABA synthesis in the tumor mass. In addition, CCRCC was homogeneously immunopositive for GAD67 throughout the tumor whereas GBM cells were immunonegative, suggesting that CCRCC cells are able to produce GABA, and that the GABA2 observed in the GBM ¹³C spectrum is derived from GABAergic interneurons (blue arrows) and GABAergic projections (green arrowheads) interspersed within the tumor tissue.

FIGURE 6.

Primary CCRCC in the kidney and the CCRCC brain metastasis show strong GAD67 immunoreactivity. The histopathological features of the CCRCC were well preserved in the metastatic brain lesion, including GAD67 immunoreactivity. This observation suggests that GABA synthesis was likely present in the primary renal mass and is not an idiosyncratic reaction to the brain microenvironment neither in the original patient or when transplanted to the mouse brain. H&E, hematoxylin and eosin.