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Authors

Seyfried, Thomas N
Marsh, Jeremy
Shelton, Laura M
et al.

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Is the restricted ketogenic diet a viable alternative to the standard of care for managing malignant brain cancer?

Thomas N. Seyfried[a,*]**, Jeremy Marsh**[a,b]**, Laura M. Shelton**[a,c]**,
Leanne C. Huysentruyt**[a,d]**, Purna Mukherjee**[a]

[a] *Biology Department, Boston College, Chestnut Hill, MA 02467, United States*
[b] *Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, United States*
[c] *Minerva Biotechnologies, Waltham, MA 02451, United States*
[d] *Department of Medicine, Hematology and Oncology, University of California, San Francisco, CA, United States*

Summary   Malignant brain cancer persists as a major disease of morbidity and mortality. The failure to recognize brain cancer as a disease of energy metabolism has contributed in large part to the failure in management. As long as brain tumor cells have access to glucose and glutamine, the disease will progress. The current standard of care provides brain tumors with access to glucose and glutamine. The high fat low carbohydrate ketogenic diet (KD) will target glucose availability and possibly that of glutamine when administered in carefully restricted amounts to reduce total caloric intake and circulating levels of glucose. The restricted KD (RKD) targets major signaling pathways associated with glucose and glutamine metabolism including the IGF-1/PI3K/Akt/Hif pathway. The RKD is anti-angiogenic, anti-invasive, anti-inflammatory, and pro-apoptotic when evaluated in mice with malignant brain cancer. The therapeutic efficacy of the restricted KD can be enhanced when combined with drugs that also target glucose and glutamine. Therapeutic efficacy of the RKD was also seen against malignant gliomas in human case reports. Hence, the RKD can be an effective non-toxic therapeutic option to the current standard of care for inhibiting the growth and invasive properties of malignant brain cancer.
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Ketogenic diet and epilepsy management

The high fat, low carbohydrate ketogenic diet (KD) has long been recognized as an effective non-toxic therapy for managing epileptic seizures in children (Freeman and Kossoff, 2010). The mechanisms by which the KD manages seizures are linked to shifts in brain energy metabolism (DeVivo

et al., 1973, 1978; Mantis et al., 2004). While glucose is the sole metabolic fuel used for nearly all brain functions under normal physiological conditions (McKenna et al., 2006), the brain will metabolize ketone bodies for energy when glucose levels become limiting, as would occur during water-only therapeutic fasting or starvation (Owen et al., 1967; VanItallie and Nufert, 2003; Cabioglu et al., 2005; Mahoney et al., 2006). It has long been known that water-only fasting is effective in managing epilepsy (Lennox and Cobb, 1928; Lennox, 1960).

The ketogenic diet was introduced as an alternative to fasting for the long-term management of seizures (Seyfried et al., 2004). The therapeutic efficacy of the KD is best when it is administered in carefully measured amounts where patients receive a fat to carbohydrate + protein ratio of 4:1 (Kossoff et al., 2009). Although anti-epileptic drugs remain as the primary therapeutic approach for seizure management, many of these drugs are toxic, costly, and ineffective against refractory seizures. As the KD is effective against refractory seizure disorders, it is now under consideration as a potential first-line anti-epileptic therapy (Kossoff, 2010). Although the KD is considered non-toxic compared to many anti-seizure drugs, toxicities have been reported in some patients and a physician's supervision is necessary in using the KD (Duchowny, 2005; Freeman and Kossoff, 2010). The KD is also gaining recognition as a potential therapy for a host of other neurological and neurodegenerative diseases including, Alzheimer's disease, Parkinson's disease, and brain cancers (Nebeling et al., 1995; Kashiwaya et al., 2000; Freeman and Kossoff, 2010; Zuccoli et al., 2010).

Standard of care for malignant brain cancer

The current standard of care for malignant brain cancer includes maximum surgical resection, radiation therapy, and chemotherapy (Mason et al., 2007; Stupp et al., 2009). Most patients with high-grade brain tumors also receive perioperative corticosteroids (dexamethasone) as part of the standard of care, which is often extended throughout the course of the disease (Koehler, 1995; Chang et al., 2005; Seyfried et al., 2010a). Despite the best available treatment, prognosis is poor for most patients with high-grade brain tumors (Davis et al., 1998; Souhami et al., 2004; Fisher and Buffler, 2005; Krex et al., 2007).

Glioblastoma multiforme (GBM)
GBM is widely considered the most malignant of brain cancers with only about 12% of patients living beyond 36 months (long-term survivors) (Krex et al., 2007; Stupp et al., 2009). GBM is heterogeneous in cellular composition consisting of tumor stem cells, mesenchymal cells, and host stromal cells (Tso et al., 2006; Ohgaki and Kleihues, 2009; Chen et al., 2010; Prestegarden et al., 2010). In addition to the neoplastic cell populations, tumor-associated macrophages/monocytes (TAM) also comprise a significant cell population in GBM sometimes equaling the number of tumor cells (Morantz et al., 1979; Phillips et al., 1982; Shinonaga et al., 1988; Nishie et al., 1999; Seyfried, 2001; Seyfried et al., 2010a). TAM indirectly contributes to tumor progression through release of pro-inflammatory and pro-angiogenic factors (Nishie et al., 1999; Seyfried, 2001; Lewis

and Murdoch, 2005; Seyfried et al., 2010a). Many of the neoplastic cells in GBM invade through the neural parenchyma well beyond the main tumor mass making complete surgical resections exceedingly rare (Kallenberg et al., 2009; Talacchi et al., 2010). There have been no major advances in GBM management for over 50 years, though use of Temozolomide has produced marginal improvement in survival (Souhami et al., 2004; Stupp et al., 2009).

We recently described how the current standard of care for GBM and other high-grade brain tumors could accelerate tumor growth thereby decreasing the probability of long-term patient survival (Seyfried et al., 2010a,b). This prediction was based on new information regarding tumor energy metabolism. It is now recognized that glucose and glutamine are the prime metabolic fuels for driving the growth of malignant tumors including brain tumors (Spence et al., 1998; Yang et al., 2009; DeBerardinis and Cheng, 2010; Seyfried et al., 2010b; Seyfried and Shelton, 2010; Shelton et al., 2010a). Indeed, some glioma cells can become addicted to glutamine (Wise et al., 2008). Hence, ready access to glucose and glutamine will accelerate tumor growth thus enhancing the probability of recurrence and reduced progression free survival.

Brain cancer energy metabolism and the standard of care: role of glutamine

In contrast to extracranial tissues where glutamine is the most available amino acid, glutamine is tightly regulated in the brain through its involvement in the glutamate—glutamine cycle of neurotransmission (McKenna et al., 2006; Hawkins, 2009). Glutamate is a major excitatory neurotransmitter that must be cleared rapidly following synaptic release in order to prevent excitotoxic damage to neurons (Takano et al., 2001; Hawkins, 2009). Glial cells possess transporters for the clearance of extracellular glutamate, which is then metabolized to glutamine for delivery back to neurons. Neurons metabolize the glutamine to glutamate, which is then repackaged into synaptic vesicles for future release (Hawkins, 2009). The glutamate—glutamine cycle maintains low extracellular levels of both glutamate and glutamine in normal neural parenchyma. Disruption of the glutamate—glutamine cycle can provide neoplastic GBM cells access to glutamine as we recently described (Seyfried et al., 2010b).

In contrast to normal glia, neoplastic glioma cells secrete glutamate. Glioma glutamate secretion is thought to contribute in part to neuronal excitotoxicity and tumor expansion (Takano et al., 2001). Neurotoxicity from mechanical trauma (surgery), radiotherapy, and chemotherapy can also increase extracellular levels of glutamate contributing further to tumor progression (Takano et al., 2001). How might information on glioblastoma energy metabolism relate to disease progression and to the standard of care for this cerebral neoplasm?

It is well documented that radiation and chemotherapies induce necrosis and inflammation, both of which phenomena will increase tissue glutamate levels (Di Chiro et al., 1988; Monje et al., 2007; Kallenberg et al., 2009; Lee et al., 2010). Local astrocytes rapidly clear extracellular glutamate metabolizing it to glutamine for release to
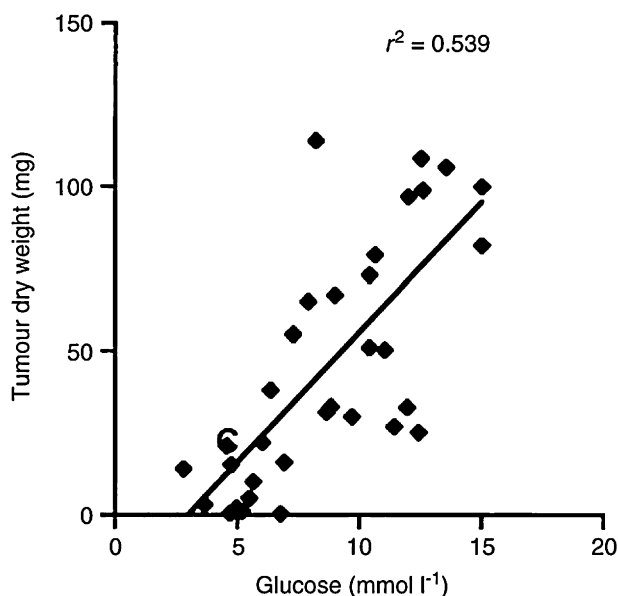
Figure 1 Linear regression analysis of plasma glucose and CT-2A tumor growth in C57BL/6 mice from both standard diet and KD dietary groups combined ($n = 34$). These analyses included the values for plasma glucose and tumor growth of individual mice from both food-restricted and unrestricted groups. The linear regression was highly significant ([*]$P < 0.001$) and indicates that circulating glucose levels are highly predictive of tumor growth rate (Seyfried et al., 2003).
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neurons. In the presence of dead or dying neurons, however, surviving tumor cells and TAM will use astrocyte-derived glutamine for their energy and growth. Radiation damage to tumor cell mitochondria will hasten a dependence on glucose and glutamine for growth and survival (Warburg, 1956a; Seyfried and Shelton, 2010). Indeed, radiation therapy is known to upregulate the PI3K/Akt signaling pathway, which drives glioma glycolysis and chemotherapeutic drug resistance (Elstrom et al., 2004; Zhuang et al., 2009; Kargiotis et al., 2010; Seyfried and Shelton, 2010).

Brain cancer energy metabolism and the standard of care: role of glucose

High-dose glucocorticoids (dexamethasone) are generally prescribed to reduce radiation-associated brain swelling and tumor edema. It is well documented that dexamethasone significantly elevates blood glucose levels (Lukins and Manninen, 2005; Hans et al., 2006; Noch and Khalili, 2009; Kargiotis et al., 2010). Glucose fuels tumor cell glycolysis as well as serving as a precursor for glutamate synthesis (Warburg, 1956a; Seyfried et al., 2003; Seyfried and Mukherjee, 2005a; McKenna et al., 2006). Using linear regression analysis, we showed that the growth rate of the CT-2A experimental astrocytoma was directly dependent on blood glucose levels (Fig. 1). The higher the glucose levels, the faster the tumors grew. As glucose levels fall, tumor size and growth rate falls. Hyperglycemia not only contributes to rapid tumor cell growth, but also enhances white matter damage in patients receiving radiation therapy (Szerlip et al., 2011). Hyperglycemia was also directly linked to poor
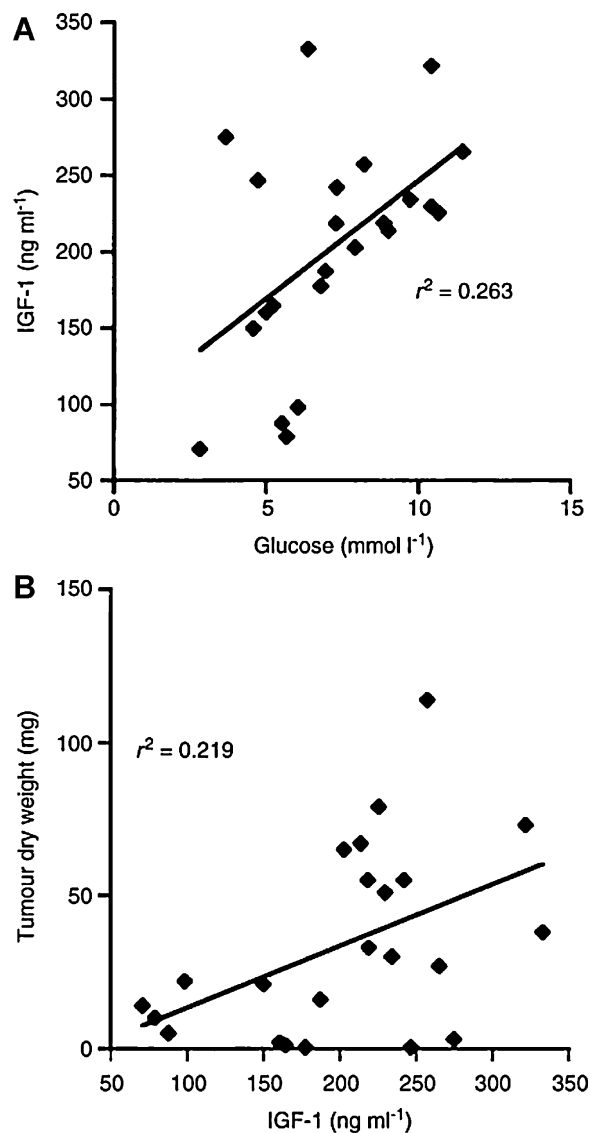

Figure 2 Linear regression analysis of plasma glucose and IGF-1 levels (A), and plasma IGF-1 levels and CT-2A-tumor growth (B). The mice used for these studies were fed with either a standard diet or a KD ($n = 23$). These analyses included the values for plasma glucose and tumor growth of individual mice from both the UR- and R-fed groups. The linear regressions were highly significant ($P < 0.01$) and indicate that circulating levels of IGF-1 and tumor growth are largely dependent on circulating levels of glucose (Seyfried et al., 2003).
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prognosis in humans with malignant brain cancer (McGirt et al., 2008; Derr et al., 2009). In other words, the findings in mice that elevated glucose accelerates brain tumor growth were corroborated in studies on humans.

Moreover, we found that the expression of insulin-like growth factor 1 (IGF-1) was also dependent on circulating glucose levels (Seyfried et al., 2003; Marsh et al., 2008a) (Fig. 2A). IGF-1 is a cell surface receptor linked to rapid tumor growth through the PI3K/Akt signaling pathway (Marsh et al., 2008a). The association of plasma IGF-1 levels with tumor growth rate is due primarily to circulating levels of

glucose (Fig. 2B). These findings in animal models and in brain cancer patients indicate that tumor growth rate and prognosis is dependent to a significant extent on circulating glucose levels. Glucose is the prime fuel for glycolysis, which drives growth of most brain cancer (Oudard et al., 1996, 1997; Seyfried and Mukherjee, 2005a). As long as circulating glucose levels remain elevated, tumor growth will be difficult to manage.

Besides serving as a metabolic fuel and for their role in the synthesis of lipids, proteins, and nucleic acids, glucose and glutamine are also important fuels for cells of myeloid linage, i.e., macrophages, monocytes, and microglia (Newsholme, 2001; Lewis and Murdoch, 2005). These fuels will act synergistically to enhance the energy metabolism and pro-inflammatory activities of TAM. TAM responds to the local tumor environment as if it were an unhealed wound and thus release pro-angiogenic growth factors (Seyfried, 2001). Some neoplastic cells in GBM might actually arise from cells of myeloid origin or from fusion hybrids between macrophages and neoplastic stem cells (Huysentruyt and Seyfried, 2010). Glutamine is an important fuel for macrophages and other cells of the immune system (Newsholme, 2001). Access to glucose and glutamine within the tumor microenvironment will create an escalating situation of biological chaos where the intrinsic properties of TAM to heal wounds will enhance the capacity of neoplastic brain tumor cells to proliferate, invade, and self-renew (Staw and Ross, 1989; Seyfried, 2001; Seyfried et al., 2010a). In other words, normally programmed cellular events become counter productive to host survival. High glucose concentrations together with unrestricted glutamine availability will provide the necessary energy metabolites for driving the escalating situation. Fig. 3 shows how radiation therapy and dexamethasone treatment can increase availability of glucose and glutamine in the tumor microenvironment.

Although the existing standard of care for malignant brain cancer will increase patient survival over the short term (months) compared to the ''no therapy'' option, we suggest that this therapeutic strategy will eventually accelerate the energy metabolism of surviving tumor cells. Moreover, the malignant phenotype of brain tumor cells that survive radiotherapy is often greater than that of the cells from the original tumor (Kargiotis et al., 2010). Treatments that increase tumor energy metabolism will facilitate tumor cell growth and survival, thus decrease long-term patient survival. That GBM patient survival has not increased substantially in more than 50 yrs supports our hypothesis (Fisher and Buffler, 2005; Stupp et al., 2009). As long as brain cancer is viewed as something other than a metabolic disease, we contend that there will be little progress in improving progression free survival. If viewed as a metabolic disease, on the other hand, we can anticipate major advances in treatment and substantial enhancement of progression free survival.

Restricted ketogenic diet (RKD) as an alternative to the standard of care for brain cancer management

Emerging evidence suggests that metabolic therapies using ketogenic diets that lower glucose levels can help retard GBM growth in younger and older patients (Nebeling et al.,
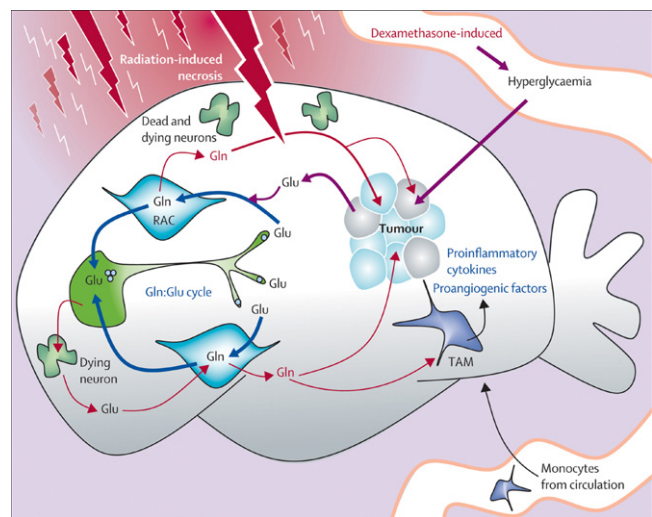

Figure 3 How the standard of care can accelerate brain tumor growth and recurrence. GBM and other high-grade brain tumors consist of multiple neoplastic cell types as well as TAMs, which release pro-inflammatory and pro-angiogenic factors. All these cells will use glucose and glutamine (Gln) as major metabolic fuels for their growth and survival. Increased glutamate (Glu) concentrations will arise after radiation/drug-induced necrosis. Reactive astrocytes (RA) take up and metabolize glutamate to glutamine, whereas hyperglycemia will arise after corticosteroid (dexamethasone) therapy. Together, these standard treatments will provide an environment that facilitates tumor cell growth, survival, and the likelihood of tumor recurrence (Seyfried et al., 2010a).
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1995; Zuccoli et al., 2010). Restricted diets are those that deliver fewer total calories in order to lower circulating glucose levels. Nebeling and co-workers first showed that the KD was an effective non-toxic management for advanced stage astrocytoma in children (Nebeling et al., 1995). Ketone bodies (b-hydroxybutyrate and acetoacetate) become an alternative fuel for brain energy metabolism when glucose levels are reduced (Veech et al., 2001; Cahill and Veech, 2003; VanItallie and Nufert, 2003; Morris, 2005). Ketone bodies might also be toxic to some human tumor cells (Magee et al., 1979; Skinner et al., 2009). Ketone bodies have known neuroprotective and anti-inflammatory action against a number of neurological and neurodegenerative diseases (Maalouf et al., 2009). Ketone body metabolism reduces oxygen free radicals while enhancing metabolic efficiency of normal cells (Veech et al., 2001; Stafford et al., 2010). It is also important to recognize that circulating ketone levels will rarely exceed 7−9 mmol in most non-diabetic patients since excess ketones will be excreted in the urine (Veech et al., 2001). Hence, ketones are considered ''good medicine'' for several neurological and neurodegenerative diseases in patients with normal physiology (Cahill and Veech, 2003; VanItallie and Nufert, 2003; Freeman and Kossoff, 2010).

Unlike normal brain cells, many tumor cells cannot metabolize ketone bodies for energy due to their various mitochondrial and genetic defects (Fredericks and Ramsey, 1978; Tisdale and Brennan, 1983; Seyfried and Mukherjee,

2005a; Zhou et al., 2007; Seyfried et al., 2010b). However, recent studies indicate that C6 glioma cells can metabolize b-hydroxybutyrate through the TCA cycle (Eloqayli et al., 2011). We recently suggested that energy generated through the TCA cycle in cancer cells could be derived from substrate level phosphorylation at the succinyl Co-A synthetase step rather than from OxPhos (Seyfried et al., 2010b; Seyfried and Shelton, 2010). Schwimmer and colleagues first showed that TCA cycle substrate level phosphorylation could compensate for genetic damage to the F1Fo ATPase and the loss of respiratory energy metabolism in yeast cells (Schwimmer et al., 2005). Substrate level phosphorylation at the succinyl Co-A synthetase step would give the appearance of normal respiration since oxygen would be consumed, CO_2 would be released, and ATP would be generated in the mitochondria, but the energy would not be derived through the F1Fo ATPase (Shelton et al., 2010c; Seyfried, 2011). We suggest that cancer cells use similar mechanisms to compensate for their dysfunctional respiration.

Moreover, the KD could potentially lower brain glutamine levels, thus restricting availability of this energy metabolite for tumor growth (Yudkoff et al., 2007; Kashiwaya et al., 2010). Previous studies with refractory pediatric astrocytoma and adult GBM suggest that ketogenic diets are therapeutically effective against malignant brain cancer (Nebeling et al., 1995; Zuccoli et al., 2010). The RKD could be even more therapeutic if combined with drugs that target glycolysis, e.g., 2-deoxyglucose or dichloroacetate (Marsh et al., 2008b; Michelakis et al., 2010). It is also possible that therapeutic synergy might occur if the type-2 diabetes drug, metformin, is administered together with the RKD (Omar et al., 2010; Oleksyszyn, 2011). By limiting the availability of glucose and glutamine to tumor cells, we suggest that RKD will improve progression free survival in patients with malignant brain cancer or most cancers for that matter. What is the evidence that supports the RKD as a novel therapy for malignant brain cancer?

Calorie restriction and the RKD are anti-angiogenic and pro-apoptotic
Angiogenesis or the vascularity of tumors is considered an important target for reducing tumor growth (Lakka and Rao, 2008). Calorie restriction has long been known to target tumor angiogenesis. Indeed, Payton Rous first suggested that restricted food intake reduced tumor growth by reducing the supporting vasculature needed for facilitating rapid tumor growth (Rous, 1914). More recent studies show that dietary energy restriction in the form of calorie restriction reduces the growth and angiogenic biomarker expression in prostate cancer and breast cancer (Mukherjee et al., 1999b; Thompson et al., 2004; Phoenix et al., 2010). The reduction in tumor vasculature was also correlated with increased apoptosis, a non-inflammatory form of programmed cell death (Mukherjee et al., 1999a,b; Lawrence and Gilroy, 2007). Apoptotic cell death differs from necrotic cell death, which is usually associated with inflammation (Lawrence and Gilroy, 2007). We suggest that apoptotic tumor cell death would be less provocative to the tumor microenvironment than would necrotic cell death. This is important since the current standard of care involving radiation therapy and temozolomide causes necrotic tumor cell death (Seyfried

et al., 2010a). In contrast to the standard of care, metabolic therapies involving calorie restriction and the RKD primarily kill tumor cells through apoptotic cell death.

We substantiated the anti-angiogenic and pro-apoptotic effects of the RKD in experimental mouse and human brain tumor models and described the molecular mechanisms of action (Mukherjee et al., 2002, 2004; Zhou et al., 2007; Seyfried et al., 2010b; Shelton et al., 2010b). It is important to mention that blood vessel structure and function is different in the tumor microenvironment than in normal microenvironment. Puchowicz et al. (2007) showed that diet induced ketosis increases capillary density in normal rat brain. In contrast to normal tissue vasculature, tumor blood vessels express leakiness and immaturity (absence of a pericyte smooth muscle sheath) (Jain, 2005; De Bock et al., 2010). Calorie restriction targets and reduces the abnormal vasculature in tumors (Mukherjee et al., 2002, 2004). We also showed the RKD caused the same phenomenon in experimental mouse and human brain tumors (Zhou et al., 2007). Our recent studies show that calorie restriction enhances expression of a-smooth muscle actin (a-SMA) in the tumor vasculature (Urits, Mukherjee, and Seyfried, unpublished observation). a-SMA is a marker for vessel maturation and integrity (Verbeek et al., 1994). Enhancement of vessel maturation in tumors could facilitate drug delivery to the tumor. Hence, calorie restriction or the RKD targets abnormal tumor blood vessels, while enhancing normal vasculature.

We found that the KD reduced brain cancer growth and angiogenesis in mice only when administered in restricted amounts. The importance of this point cannot be overemphasized, as unrestricted KD administration was largely without effect on the growth of the CT-2A astrocytoma (Seyfried et al., 2003, 2010b; Zhou et al., 2007). Blood glucose levels remain high and ketones are largely excreted in the urine when the KD is fed to mice in unrestricted amounts. Harik et al. (1997) also showed that unrestricted feeding of the KD had no significant effect on brain glucose metabolism in rats. However, Scheck and colleagues reported growth inhibition of the mouse GL261 cells from an unrestricted KD suggesting that some tumors might be susceptible to KD growth inhibition without calorie restriction or glucose reduction (Stafford et al., 2010). In contrast, we found that unrestricted consumption of the KD was ineffective in reducing tumor growth or angiogenesis (Seyfried et al., 2003; Zhou et al., 2007). The unrestricted feeding of the KD prevented glucose and ketones from reaching the therapeutic levels necessary for blocking angiogenesis and enhancing tumor cell apoptosis (Seyfried et al., 2003; Zhou et al., 2007). The importance of calorie restriction for management of CT-2A astrocytoma growth using either a standard high carbohydrate diet or a KD is illustrated in Fig. 4. Similar results were obtained with the U87MG experimental human glioma cell line (Mukherjee et al., 2004; Zhou et al., 2007).

Calorie restriction targets nuclear factor κB (NF-κB) and is anti-inflammatory
Phosphorylation and activation of NF-κB results in the transactivation of many genes including those encoding cycloxygenase-2 (COX-2) and allograft inflammatory factor-1 (AIF-1), both of which are primarily expressed
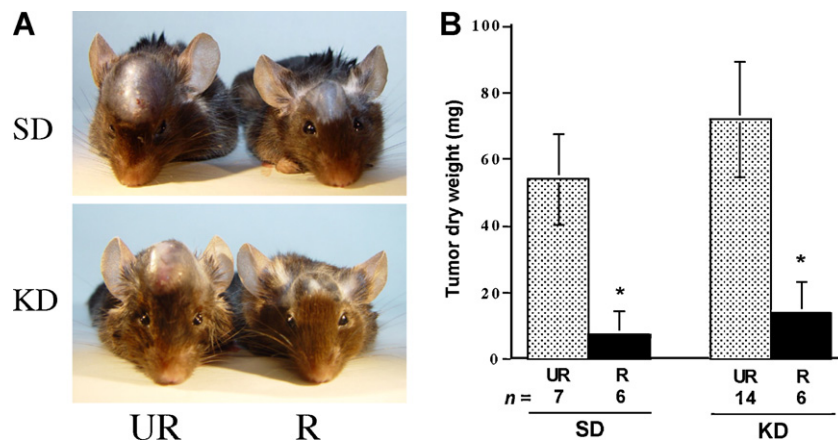
Figure 4 Influence of diet on the intracerebral growth of the CT-2A brain tumor. The visual representation (A) and quantitative assessment (B) of the tumor growth in C57BL/6J mice receiving the standard high carbohydrate diet (SD) or the ketogenic diet (KD) under either unrestricted (UR) or restricted (R) feeding as we described (Seyfried et al., 2003). Values in B are expressed as means with 95% confidence intervals, and $n =$ the number of mice examined in each group. The dry weights of the tumors in the R-fed mice were significantly lower than those in the unrestricted UR-fed mice at $P < 0.01$. The results show that DR significantly reduces tumor growth whether the mice are fed an SD or are fed a KD. No adverse effects were seen in the mice maintained on the 30—40% DR. Body weights were similar for mice in both UR groups and were reduced similarly in both R groups (Seyfried et al., 2003). Despite a reduction in total body weight, the R-fed mice were more healthy and active than the UR-fed mice as assessed by ambulatory and grooming behavior. No signs of vitamin or mineral deficiency were observed in the DR-fed mice according to standard criteria for mice. These findings are consistent with the well-recognized health benefits of mild to moderate diet restriction in rodents and in humans (Mukherjee et al., 2002; Seyfried and Mukherjee, 2005b).

by inflammatory and malignant cancer cells within the tumor microenvironment. Activated NF-κB translocates to the nucleus, binds to DNA, and then activates a number of pro-inflammatory molecules including COX-2, TNF-a, IL-6, IL-8, and MMP-9 (Karin, 2006; Atkinson et al., 2009). COX-2 enhances inflammation and promotes tumor cell survival (Portnow et al., 2002; Badie et al., 2003). We recently demonstrated that the p65 subunit of NF-κB was expressed constitutively in the CT-2A astrocytoma compared with contra-lateral normal brain tissue (Mulrooney et al., 2011). NF-κB also appears to activate mitochondrial glutaminase, which hydrolyzes glutamine to glutamate (Wang et al., 2010). Glutamate is used as an energy metabolite for tumor growth and, when secreted, can enhance tumor progression (Takano et al., 2001; Seyfried et al., 2010a). Hence, inhibition of NF-κB activation would help reduce rapid tumor growth and progression.

We showed that calorie restriction; (a) reduces the phosphorylation and degree of transcriptional activation of the NF-κB-dependent genes COX-2 and AIF-1 in CT-2A tumor tissue and, (b) reduces expression of pro-inflammatory markers lying downstream of NF-κB, e.g., macrophage inflammatory protein-2 (MIP-2) (Mulrooney et al., 2011). On the whole, we showed that the NF-κB inflammatory pathway is constitutively activated in the CT-2A astrocytoma and that CR targets this pathway and inflammation. As the inhibitory effects of CR on CT-2A growth are similar to those of the RKD, we are certain that RKD would also target glioma inflammation through similar pathways. There are no oncology drugs to our knowledge that can simultaneously target inflammation and angiogenesis while, at the same time, killing tumor cells through an apoptotic mechanism.

Calorie restriction inhibits invasion of murine GBM

Distal invasion of malignant glioma cells is often responsible for the failure of most current therapies to manage the disease. The invasive properties of many malignant human brain tumors follow the ''secondary structures of Scherer'', which include diffuse parenchymal invasion, perivascular growth, subpial surface growth, and growth along white matter tracts (Scherer, 1940; Zagzag et al., 2008; Seyfried et al., 2010b; Shelton et al., 2010d). We recently showed that the VM-M3 invasive glioblastoma model, which was derived from a spontaneous brain tumor in the VM inbred strain, is the only syngeneic mouse brain tumor to our knowledge that expresses the full complement of Scherer's secondary structures (Seyfried et al., 2010b; Shelton et al., 2010d). This model is therefore ideally suited for testing therapies that can target glioma invasion (Shelton et al., 2010b).

As seen in Fig. 5, calorie restriction reduced the growth and local invasion of the VM-M3 primary tumor. Compared to the diffuse, ill-defined border of the VM-M3 tumor observed in the unrestricted control mice, the tumor grown the CR mice appeared denser with a more defined border indicative of reduced invasion.

In addition to inhibiting local invasion, CR also reduced the distal invasion of tumor cells from the implanted ipsilateral cerebral hemisphere into the contralateral hemisphere. The VM-M3 tumor cells were engineered to express luciferase, which generates bioluminescence (VM-M3/Fluc) (Shelton et al., 2010b,d). Bioluminescent tumor cells facilitate quantitative assessment of distal invasion. While invading tumor cells were identified in all regions of the contralateral hemisphere of the control ad libitum-fed (AL) mice, only subpial invasion was found in the contralateral
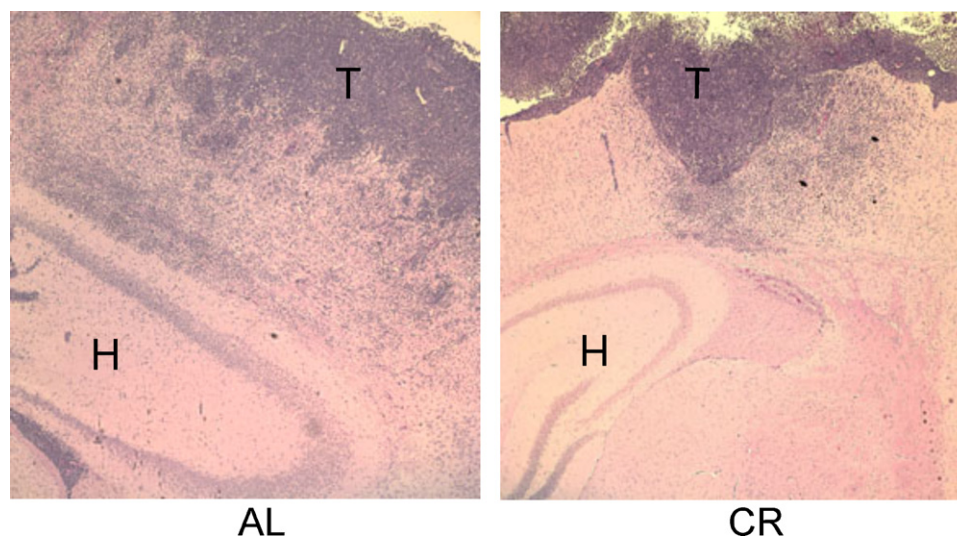
Figure 5 Influence of calorie restriction on VM-M3/Fluc tumor growth.
VM-M3/Fluc tumor fragments were implanted into the cerebral cortex, were fixed, and then stained with haematoxylin and eosin (H&E) as described (Shelton et al., 2010b). Images are shown at $50\times$ (T = tumor, H = Hippocampus). Tumor cell invasion through the neural parenchyma (dark blue cells) is greater in the in the *ad libitum* AL-fed mice than in the CR-fed mice. The boarder between tumor tissue and normal brain tissue is less sharp in the AL-fed mice than in the CR-fed mice. The results show that CR reduces VM-M3/Fluc tumor cell invasion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Reprinted with permission from ASN Neuro (Shelton et al., 2010b).

hemisphere of the CR group (Fig. 6). The total percentage of Ki-67-stained cells within the primary tumor and the total number of blood vessels was also significantly lower in the CR-treated mice than in the mice fed AL, indicating that CR is also anti-proliferative and anti-angiogenic in this invasive tumor model (Seyfried et al., 2010b; Shelton et al., 2010b).

These findings clearly indicate that calorie restriction alone can inhibit proliferation as well as both local and distal invasion of the VM-M3 mouse GBM. We would also expect similar findings using the RKD. Hence, therapies that can restrict availability of glucose and glutamine will be effective in targeting the most malignant properties of the disease.
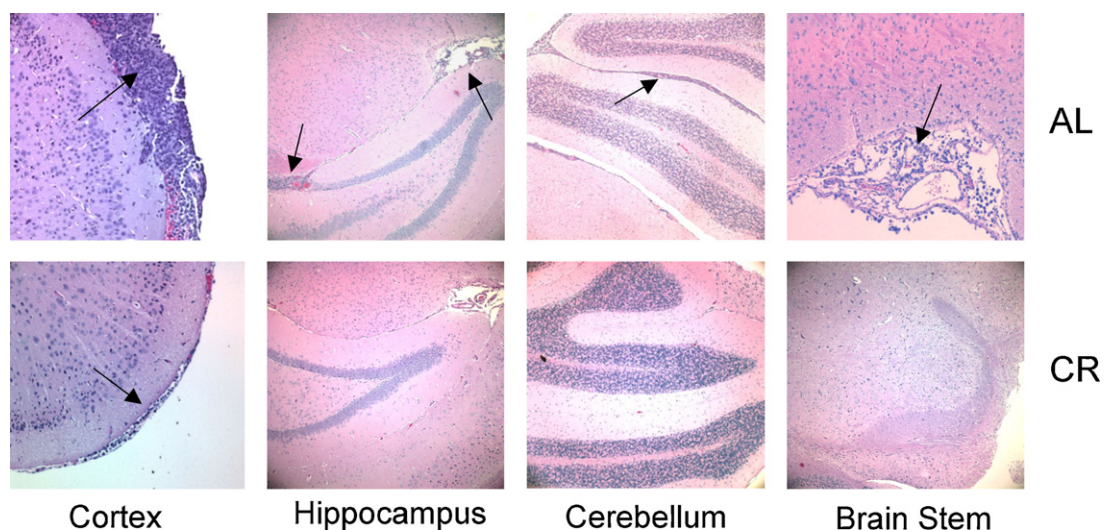

Figure 6 Influence of calorie restriction on VM-M3/Fluc tumor cell invasion to the contralateral hemisphere. VM-M3/Fluc tumor fragments were implanted into the right cerebral hemisphere as described (Shelton et al., 2010b). Histological analysis (H&E) was used to validate the presence of tumor cells under AL (top) and CR (bottom) in cerebral cortex ($200\times$), hippocampus ($100\times$), cerebellum ($100\times$), and brain stem ($200\times$) of the contralateral hemisphere. Arrows indicate the presence of tumor cells. At least three samples were examined per group.
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Dietary caloric adjustments and brain cancer management

The dietary conditions we used for managing brain cancer in mice are similar to the conditions we used for managing epileptic seizures in the EL mouse, a natural model of idiopathic generalized epilepsy (Mantis et al., 2004). Seizures were unmanageable in EL mice when they were fed the KD in unrestricted amounts, but seizures were almost completely managed when the diet was given in restricted amounts to reduce glucose levels. It is also recognized that seizure management in patients with epilepsy is best when the KD is administered in carefully measured amounts rather than when consumed in unrestricted or unmeasured amounts (Freeman et al., 2000, 2007a,b; Freeman and Kossoff, 2010). Indeed, the KD can be ineffective for seizure management in those children who gain significant weight on the diet (J. Freeman, personal communication). The therapeutic efficacy of the KD against brain cancer in mice and humans is directly correlated with changes in circulating levels of glucose and ketone bodies (β-OHB) (Nebeling et al., 1995; Seyfried et al., 2008, 2010b; Zuccoli et al., 2010). The reduced glucose levels are also associated with some body weight reduction, as CR reduces body weight.

It is important to recognize that calories are metabolized differently among individual people and among individual mice of the same strain. It can therefore be difficult to induce metabolic stress on brain tumors by adjusting calories alone whether for humans or for mice. This is why we used body weight as the independent variable for adjusting the degree of calorie restriction (Zhou et al., 2007). Some people might achieve this metabolic state without significant weight reduction, while others might require some weight reduction to achieve the metabolic state. We know from the results with two children and an adult with malignant brain cancer that tumor growth can be reduced if blood glucose is lowered and ketones are elevated (Nebeling et al., 1995; Zuccoli et al., 2010). We know from our work in mice that tumor growth is rapid if glucose is not lowered despite elevations in ketones and normal body weight (Seyfried et al., 2003; Zhou et al., 2007). The degree of calorie adjustment might vary considerably from one person to the next in order to achieve the metabolic state required to reduce brain tumor growth. Considering that personalized therapy is the new mantra for cancer management (Hayden, 2009), we suggest that the degree caloric adjustment can be personalized for achieving maximum therapeutic benefit.

Although blood glucose levels are similarly reduced under the RKD and calorie restriction, we think the RKD will be more therapeutic against brain cancer than calorie restriction alone. This prediction comes from our findings showing that circulating ketone levels are higher with the RKD than with CR alone (Mantis et al., 2004; Zhou et al., 2007). The higher level of ketones achieved with the RKD than with CR alone will provide normal brain cells with adequate energy, while creating greater metabolic stress on tumor cells (Seyfried et al., 2010b). Fig. 7 illustrates the relationship between brain cancer management and circulating levels of glucose and ketones. Elevated ketones will reduce glycolysis especially when circulating glucose levels are reduced (Kashiwaya et al., 1994, 2010; Katayama et al., 1994; Puchowicz et al., 2005). Tumor cells have a greater
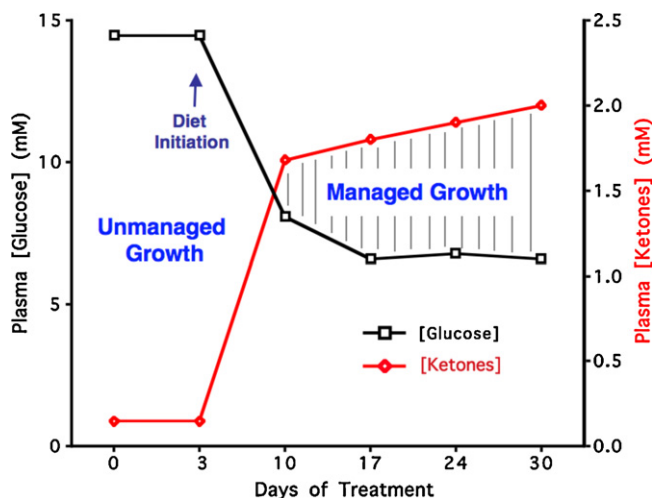

Figure 7 Relationship of circulating levels of glucose and ketones (β-hydroxybutyrate, β-OHB) to brain tumor management. The glucose and ketone values are within normal physiological ranges under fasting conditions in mice and produce anti-angiogenic, anti-inflammatory, and pro-apoptotic effects. We refer to this state as the zone of metabolic management. Metabolic stress will be greater in tumor cells than in normal cells when mice or humans with brain cancer are in the metabolic zone. The glucose and ketone levels predicted for brain tumor management in human brain cancer patients are 3.1—3.8 mM and 2.5—7.0 mM, respectively (Seyfried and Mukherjee, 2005a; Seyfried et al., 2010b). See text for details.

dependence on glycolysis for energy and survival than normal cells, which transition to ketones for survival when glucose is lowered. We also consider that the stress placed on brain cancer patients associated with dietary caloric adjustments will be less than the stress placed on patients associated with exposure to harmful radiation and toxic drugs. In contrast to the current standard of care for brain cancer, the RKD will target tumor cell energy metabolism without harming normal cells or tissues. It is also well documented that general health is better when less total calories are consumed as part of a daily lifestyle than when more total calories are consumed (Shelton, 1974).

As long as patients can maintain their circulating glucose and ketone levels within the ''metabolic zone'', their brain tumor growth can be slowed. The metabolic zone for humans involves a glucose range of 55—65 mg/dl and a ketone range of 3—5 mmol. Tumor cells will experience considerable metabolic stress when glucose and ketones reach these levels. Moreover, demand for glucose will increase throughout the brain, as the GLT-1 transporter becomes upregulated in normal brain cells under caloric restriction (Marsh et al., 2008a). In contrast to the glucose GLT-1 transporter up-regulation in normal brain cells, GLUT-1 transporter expression is down regulated in response to glucose reduction in the tumor cells. This is opposite to the effect seen in normal cells (Marsh et al., 2008a). Under these conditions, tumor cells must now compete with normal cells for the reduced amounts of glucose. Tumor cells will starve in this competition because they are unable to use

ketone bodies as an alternative fuel due to their mitochondrial dysfunction. In light of these findings, the RKD therapy might therefore be viewed as a type of metabolic surgery. By reducing tumor size, inflammation, and angiogenesis, we also suggested that the RKD could enhance surgical resection of brain tumors (Seyfried et al., 2010b).

Synergistic interaction of RKD and 2-deoxyglucose (2-DG)

Although dietary energy restriction is effective in reducing brain tumor growth and invasion, this therapeutic approach alone is unlikely to completely eradicate all types of malignant brain tumors (Seyfried et al., 2010b; Shelton et al., 2010a). We think that metabolic diet therapy could be enhanced when combined with drugs that also target energy metabolism. Support for this hypothesis comes from our recent pilot study showing that the non-metabolizable glycolysis inhibitor, 2-deoxy-D-glucose (2-DG), worked synergistically with the RKD to reduce CT-2A astrocytoma growth (Marsh et al., 2008b). 2-DG is readily transported into cells, is phosphorylated by hexokinase, but cannot be metabolized further and thus accumulates in the cell (Aft et al., 2002). This leads to ATP depletion and the induction of cell death. In this regard, 2-DG has been described as a CR-mimetic, i.e., a drug that mimics some aspects of calorie restriction (Zhu et al., 2005; Kang and Hwang, 2006). However, treatment of animal models and cancer patients with relatively high doses of 2-DG (greater than 200 mg/kg) was largely ineffective in managing tumor growth (Landau et al., 1958; Dills et al., 1984; Cay et al., 1992). Adverse effects of 2-DG included elevated blood glucose levels, progressive weight loss with lethargy, and behavioral symptoms of hypoglycemia (Landau et al., 1958; Dills et al., 1984; Cay et al., 1992; Singh et al., 2005; Pelicano et al., 2006). These findings indicate that 2-DG alone is ineffective as a viable therapy for most cancers.

Few studies have evaluated the therapeutic efficacy of anti-glycolytic or anti-cancer drugs in combination with calorie-restricted diets (Seyfried et al., 2010b). Recent studies suggest that calorie restriction and fasting can enhance the therapeutic action of anti-cancer drugs (Raffaghello et al., 2008; Safdie et al., 2009; Seyfried et al., 2010b). We showed that the KD-R supplemented with 25 mg/dl of 2-DG was effective in reducing intracerebral tumor growth to a greater extent than was either 2-DG or the KD-R when administered alone, indicating that a synergistic interaction occurred between 2-DG and the diet (Fig. 8).

Some toxicity was seen in mice treated with the drug diet combination, as several mice died when given the combination. This was surprising since no toxicity was observed in mice that received either therapy alone. It is unclear whether a similar phenomenon would occur in humans using this drug/diet combination. However, the approximate LD50 for 2-DG in humans is about 350 mg/kg (Singh, 2006; Dwarakanath, 2009; Dwarakanath et al., 2009). The toxicity seen in mice might relate to their high basal rate, which is about 7-fold greater than that in humans (Mahoney et al., 2006; Marsh et al., 2008b).

In light of the findings in the mouse study, low 2-DG doses (20—30 mg/kg) should be used initially when combining 2-DG with the RKD for cancer treatment in humans. We proposed that energy stress on tumor cells would be greater in individuals receiving the drug/diet combination than in individuals receiving either therapy alone (Marsh et al., 2008b; Seyfried et al., 2010b). It is our opinion that none of the current drugs considered calorie restriction mimetics will show major therapeutic effect against glioblastoma if used in the absence of some degree of calorie restriction (Omar et al., 2010). Based on our findings and those from the Longo group (Raffaghello et al., 2008; Safdie et al., 2009), we suggest that the therapeutic efficacy of many anti-cancer drugs could be enhanced when administered in combination with energy-restricted diets.

It is important to mention that CR alone does not directly target glutamine, and might therefore be less effective than the RKD in managing the growth of tumors that depend more on glutamine than on glucose (Shelton et al., 2010a). The RKD might have some effect in reducing brain glutamine levels (Yudkoff et al., 2007; Kashiwaya et al., 2010). We also showed that the glutamine analogue, 6-diazo-5-oxo-L-norleucine (DON), was effective in reducing the systemic metastatic spread of the VM-M3 tumor (Shelton et al., 2010a). DON targets glutamine uptake into cells, but can be toxic (Shelton et al., 2010a). Phenylbutyrate is less toxic than DON and could also be effective in reducing blood glutamine levels. Phenylbutyrate is metabolized to phenylacetate, which then binds to glutamine for excretion. AN-113, a novel pro-drug of 4-phenylbutyrate, could be better than phenylbutyrate in reducing brain glutamine levels since blood—brain barrier permeability is considered better for AN-113 than for phenylbutyrate (Entin-Meer et al., 2007). Although GBM is not generally considered metastatic, there are a number of reports showing that GBM can be highly metastatic if the tumor cells gain access to the circulation (Hoffman and Duffner, 1985; Taha et al., 2005; Gotway et al., 2011; Zhen et al., 2010; Lun et al., 2011). Hence, the RKD combined with drugs that also target glucose and glutamine could be effective in controlling GBM that grows both within and outside the CNS.

The findings in mouse brain tumors exemplify the efficacy and versatility of reduced calorie intake as a broad-spectrum inhibitor of malignant glioma growth and suggest that dietary energy restriction may extend survival in patients with advanced brain cancers. CR and RKD will simultaneously target multiple metabolic pathways in tumor cells without causing adverse effects or toxicity to normal cells (Marsh et al., 2008a; Seyfried et al., 2010b; Shelton et al., 2010b; Zuccoli et al., 2010). The systemic energy transition from glucose to ketones will reduce inflammation in the tumor microenvironment, thus reducing progression as we recently described (Mulrooney et al., 2011).

Basically, dietary energy restriction and ketone body metabolism delays entropy, which is the bioenergetic signature of cancer (Seyfried and Shelton, 2010). Entropy refers to the degree of disorder in systems and is the foundation of the second law of thermodynamics. Szent-Gyorgyi described cancer as an increased state of entropy, where randomness and disorder predominate (Szent-Gyorgyi, 1977). The RKD metabolic therapy could be even more effective when
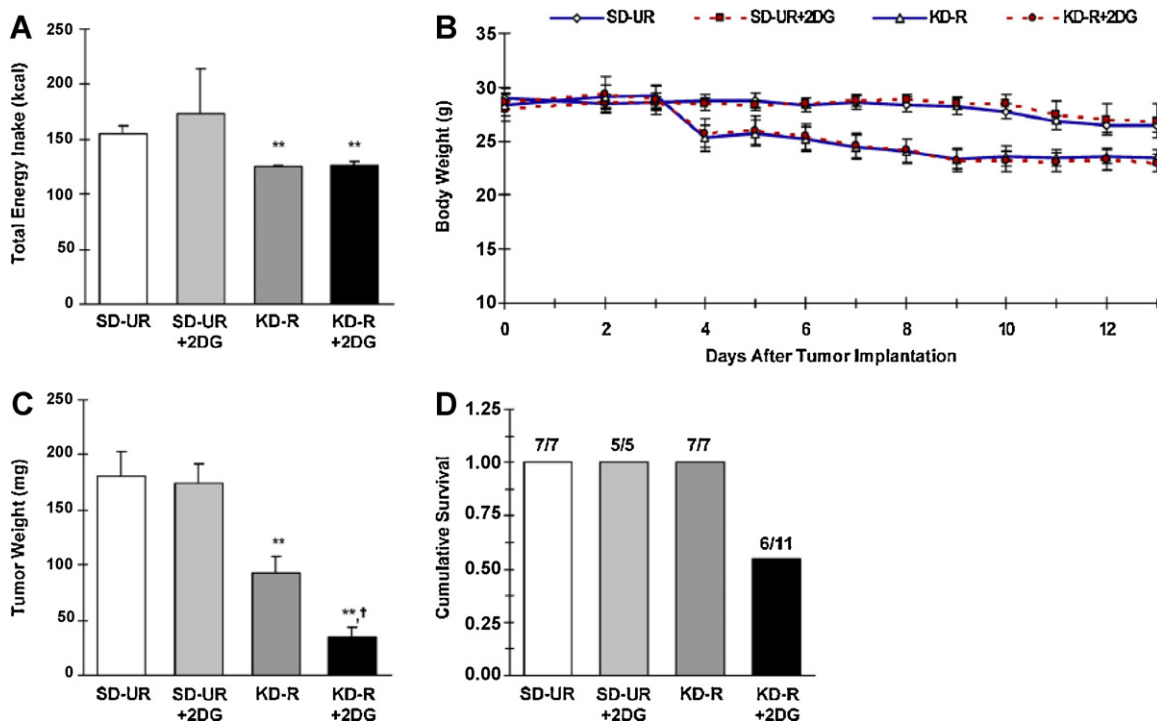
Figure 8 Influence of the restricted ketogenic diet with or without 2-Deoxyglucose (2-DG) on total energy intake (A), body weight (B), tumor growth (C), and on cumulative survival (D) in mice bearing the orthotopically implanted CT-2A malignant astrocytoma. All mice were fed the standard high carbohydrate rodent diet (SD) in unrestricted (UR) amounts for the first 3 days after tumor implantation prior to their separation into one of four diet groups ($n = 5-11$ mice/group) fed either SD-UR or a KD-R with or without 2-DG (25 mg/kg) for 10 days. The average total energy intakes in (A) represent the number of kcals consumed by the indicated group over the dietary treatment period (days 3—13). The four groups were matched for body weight. 2-DG was initiated 6 days after tumor implantation and was continued for 7 days (B and C). As shown in (B), the feeding paradigm for the KD-R and KD-R + 2-DG groups was designed to reduce body weights by ~20% relative to values recorded before the diet was initiated (3 days after tumor implantation). All values are expressed as the mean ± S.E.M. In (A&C), average values for the indicated group are significantly less than the average value for the SD-UR group at [**]$P < 0.01$. The mean value for the KD-R + 2-DG group is significantly lower than the mean value for the KD-R group at [†]$P < 0.01$. No significant differences were observed between the SD-UR and SD-UR + 2-DG groups throughout the study. For (D), the number of tumor-bearing mice that were alive in each group at the conclusion of the study is listed as a ratio above each solid vertical bar (e.g. the ''6/11'' indicates that 6 of the 11 original mice were alive at the end of the study in the associated group). The findings indicate that 2-DG acted synergistically with the restricted KD to reduce tumor growth. Reprinted with permission from Nutrition & Metabolism.

combined with drugs that also target energy metabolism. Hence metabolic therapies, which lower glucose availability and elevate ketone bodies, can reduce brain tumor growth through integrated anti-angiogenic, anti-invasive, and pro-apoptotic mechanisms.

Does the restricted ketogenic diet represent a viable option for managing malignant brain cancer?

The answer to this question should be framed in light of what we know about the origin of cancer. Substantial evidence collected from numerous investigators over many years indicates that mitochondrial abnormalities are the hallmark of all cancers including brain cancer. These mitochondrial abnormalities compromise energy production through OxPhos (Roskelley et al., 1943; Pedersen, 1978; Villalobo and Lehninger, 1979; Carew and Huang, 2002; Cuezva et al., 2002; Ramanathan et al., 2005; Arismendi-Morillo and Castellano-Ramirez, 2008; Arismendi-Morillo, 2009; Bayley

and Devilee, 2010; Seyfried et al., 2010b; Seyfried and Shelton, 2010). The work of Arismendi-Morillo showed that the ultra structure of normal tissue mitochondria differs markedly from the ultra structure of mitochondria in GBM and other malignant astrocytomas (Arismendi-Morillo and Castellano-Ramirez, 2008; Arismendi-Morillo, 2009, 2010). In contrast to normal mitochondria, which contain numerous cristae, mitochondria from GBM tissue samples showed swelling with partial or total cristolysis (Fig. 9). Cristae contain the proteins of the respiratory complexes, and play an essential structural role in facilitating energy production through OxPhos (Galluzzi et al., 2010). The structural defects in human glioma mitochondria are also consistent with lipid biochemical defects in murine gliomas (Kiebish et al., 2008, 2009). In addition to these findings, Poupon and colleagues also indicated that the high glycolytic activity seen in malignant gliomas could arise from mitochondrial structural abnormalities (Oudard et al., 1997). Hence, substantial evidence exists showing that respiratory capacity is defective in gliomas.
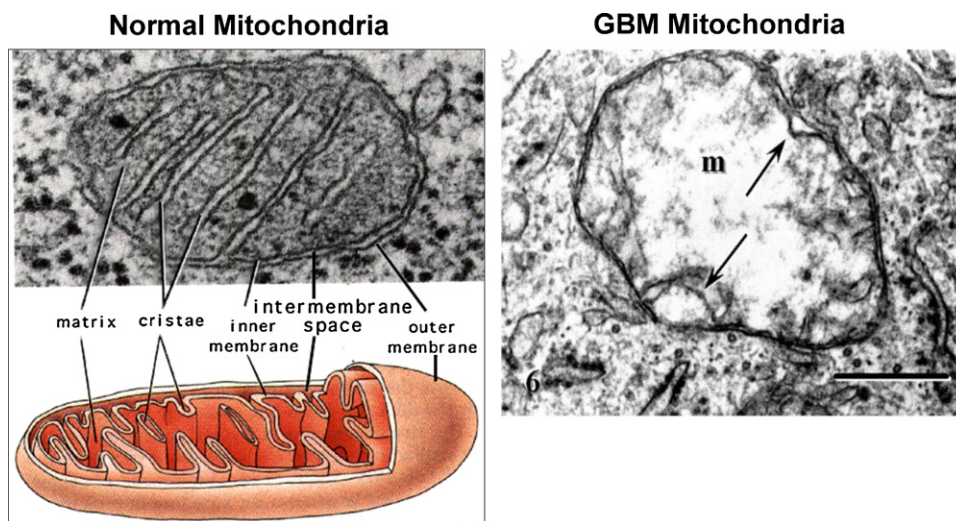
Normal Mitochondria **GBM Mitochondria**

Figure 9 Typical ultrastructure of a normal mitochondrion and a mitochondrion from GBM tissue. Normal mitochondria contain elaborate cristae, which are extensions of the inner membrane and contain the protein complexes of the electron transport chain necessary for producing ATP through OxPhos. The mitochondrion from the GBM (m) is enlarged and shows a near total breakdown of cristae (cristolysis) and an electron-lucent matrix. The absence of cristae in GBM mitochondria indicates that OxPhos would be deficient. The arrow indicates an inner membrane fold. Bar: 0.33 μm. Method of staining: uranyl acetate/lead citrate. The GBM mitochondria was reprinted with permission from *Journal of Electron Microscopy* (Arismendi-Morillo and Castellano-Ramirez, 2008). The normal mitochondria and diagram were from http://academic.brooklyn.cuny.edu/biology/bio4fv/page/mito.htm.

Based on their numerous findings in human glioma cell lines and tissues, the Poupon and Arismendi-Morillo groups suggested that the majority of astrocytomas are incapable of producing adequate amounts of energy through oxidative phosphorylation (Oudard et al., 1995, 1996, 1997; Arismendi-Morillo and Castellano-Ramirez, 2008; Arismendi-Morillo, 2009, 2010). Besides these ultrastructure findings, Renner et al. (2010) showed that tumor cells isolated from human GBM could produce ATP in the presence of potassium cyanide. Cyanide blocks cytochrome c oxidase and kills normal control cells, which obtain energy through OxPhos. Mitochondrial energy production in the presence of cyanide suggests that OxPhos is not likely the origin of the energy produced in these GBM cells. These and other studies suggest that OxPhos is deficient in malignant gliomas and that energy through oxidative metabolism alone would be incapable of maintaining viability in glioma cells (Seyfried and Mukherjee, 2005a).

Otto Warburg first proposed that all cancers arise from irreversible damage to cellular respiration (Warburg, 1931, 1956a). Although confusion has surrounded Warburg's theory (Koppenol et al., 2011), the theory has never been formally disproved and remains a credible explanation for the origin of tumor cells (Cuezva et al., 2004; Ferreira, 2010; Seyfried and Shelton, 2010). We recently described how the appearance of OxPhos in tumor cells could represent ''pseudo respiration'' that could arise in part from mitochondrial glutamine fermentation and non-oxidative substrate level phosphorylation within the tumor cell mitochondria (Seyfried et al., 2010b; Seyfried, 2011). Besides glucose, glutamine is recognized as a major fuel for many cancer cells including glioma (Seyfried and Mukherjee, 2005a; Wise et al., 2008; Dang, 2010; Seyfried et al., 2010b; Seyfried and Shelton, 2010; Shelton et al., 2010c). Under pseudo respiration, O_2 is consumed and mitochondrial CO_2 and ATP are produced. However, most of the mitochondrial ATP production in tumor mitochondria can arise from substrate level phosphorylation at the succinyl-Co-A synthetase step in the TCA cycle rather than from normal OxPhos. We recently presented evidence showing how VM-M3 tumor cells could generate ATP through a non-oxidative mechanism involving substrate level phosphorylation in the TCA cycle (Shelton et al., 2010c; Seyfried, 2011). It is not yet clear whether this phenomenon is specific to these mouse brain cancer cells or is a more general phenomenon seen in all cancer cells.

As the result of damaged respiration, cancer cells must rely on non-oxidative energy metabolism to maintain viability. Besides TCA cycle substrate level phosphorylation, aerobic glycolysis or the ''Warburg effect'' also plays a role in producing energy through substrate level phosphorylation in the cytoplasm (Warburg, 1956b; Seyfried et al., 2010b; Seyfried and Shelton, 2010; Shelton et al., 2010c). Aerobic glycolysis arises from damaged respiration. A protracted reliance on non-oxidative energy metabolism, involving glucose and amino acid fermentation with substrate level phosphorylation, can cause genomic instability and other recognized hallmarks of cancer (Seyfried et al., 2010b; Seyfried and Shelton, 2010; Seyfried, 2011). It appears that the function of DNA repair enzymes and the integrity of the nuclear genome is dependent to a large extent on the energy derived from normal respiration (Delsite et al., 2003; Rasmussen et al., 2003; Kulawiec et al., 2008; Smiraglia et al., 2008; Lu et al., 2009; Veatch et al., 2009; Chandra and Singh, 2010; Yang et al., 2010). In other words, the Warburg effect and genomic instability ultimately arise from damage to OxPhos. Whether respiratory damage is irreversible as Warburg suggested or might be reversed remains spec-

ulative. It is difficult to imagine, however, how respiration or the mitochondria cristolysis seen in GBM could be easily reversed (Fig. 9).

Many tumors become resistant to chemotherapy because they have access to glucose and glutamine, which drive their fermentation and enhance their resistance to apoptotic death. Indeed, glycolytic pyruvate activates the p-glycoprotein. The p-glycoprotein exports drugs out of cells and is largely responsible for the resistance of tumor cells to chemotherapy (Aller et al., 2009; Wartenberg et al., 2010). Hence, targeting glucose and glutamine availability becomes a rational approach to the management of most cancers including brain cancer.

Most current therapies used for brain cancer management do not directly target energy metabolism. In fact, the recommended standard of care for brain cancer management enhances brain tumor energy metabolism as we recently described (Seyfried et al., 2010a) (Fig. 3). How is it possible to effectively manage the disease if the recommended treatments provoke the disease? It should not therefore be surprising that few patients with malignant brain cancer experience long-term progression free survival after receiving the current standard of care.

It is interesting to note that none of the current approaches to brain cancer management discussed at a recent symposium involved strategies to target tumor cell energy metabolism (Yang and Liau, 2010). Several presentations at this symposium discussed the failures associated with current approaches to management. As long as brain cancer is viewed as something other than a disease of energy metabolism, the failures will likely continue in our opinion. In contrast to most current brain cancer therapies, CR and the RKD are the only therapeutic approaches that simultaneously target energy metabolism, angiogenesis, and inflammation through the IGF-1-PI3K-Akt-Hif-1a signaling (Mukherjee et al., 2002, 2004; Marsh et al., 2008a; Seyfried et al., 2010b; Mulrooney et al., 2011). The therapeutic efficacy of the RKD can be enhanced especially when combined with drugs that further reduce availability of glucose and glutamine.

According to the evidence presented here, the RKD can represent a viable non-toxic option for managing malignant brain cancer. The RKD can target tumor cells globally without harming normal neurons and glia. The blood—brain barrier is less of an issue with the RKD therapy than with conventional therapies. Although the RKD therapy is more rational than the current standard of cancer for malignant brain cancer, the RKD is not without shortcomings. Compliance can be a major obstacle in attempting to implement the RKD. Some people can have difficulty in maintaining blood glucose and ketones in the ranges needed to target angiogenesis and to control tumor growth. Considerable patient discipline and motivation is required for implementing the RKD as a therapy. Many neurooncologists are also unfamiliar with the principles and concepts about how the therapy controls tumor growth. Consequently, some patients might be discouraged from using the RKD. Nevertheless, we remain hopeful that the metabolic approach to brain cancer management, using the RKD together with drugs that target glucose and glutamine, will eventually become an effective therapeutic option to the current standard of care for brain cancer management.

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