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LAB-METABOLIC PATHWAYS

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Abstracts

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MP-01. ASTROCYTE-ELEVATED GENE-1 (AEG-1) IS INDUCED BY HYPOXIA AND GLUCOSE DEPRIVATION AND MODULATES THE GLYCOLYTIC PHENOTYPE IN GLIOBLASTOMA

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Glioblastoma continues to rank among the most lethal primary human tumors. Despite treatment with the most rigorous surgical, chemotherapeutic, and radiation regimens, the median survival is just 12-15 months after diagnosis for patients with glioblastoma. One feature of glioblastoma associated with poor prognosis is the degree of hypoxia and expression levels of hypoxia-inducible factor-1 α (HIF-1α). HIF-1α expression allows metabolic adaptation to low oxygen availability, partly through upregulation of vascular endothelial growth factor (VEGF) and increased tumor angiogenesis. In this study, we demonstrate an induced level of astrocyte-elevated gene-1 (AEG-1) in high-grade as compared to low-grade astrocytomas and association of AEG-1 with necrotic areas in glioblastoma. AEG-1 was recently demonstrated to be an oncogene that can induce angiogenesis in glioblastoma. Results from in vitro studies show that AEG-1 is induced by hypoxia in a HIF-1α-dependent manner and that PI3K inhibition abrogates AEG-1 induction during hypoxia. Furthermore, we show that AEG-1 is induced by glucose deprivation and that prevention of intracellular reactive oxygen species (ROS) accumulation prevents this induction. Additionally, AEG-1 knockdown results in increased, whereas AEG-1 overexpression results in decreased, ROS production and glucose deprivation-induced cytotoxicity, indicating that AEG-1 induction is necessary for cells to survive this type of stress. Moreover, AEG-1 modulates the expression of glycolytic enzymes in glioblastoma cells in vitro and in vivo and regulates the expression of these enzymes as well as glycolytic flux during metabolic stress, such as glucose deprivation. The AEG-1-induced glycolytic profile in glioblastoma cells is also modulated by glycolytic inhibition. Studies in nude mice demonstrate that AEG-1 knockdown reduces the growth of glioblastoma xenografts and also promotes chemosensitivity to glycolytic inhibition in vitro. These findings identify a novel role for AEG-1 in the regulation of glycolysis in glioblastoma and indicate that anti-glycolytic therapies may be useful in treating malignancies that demonstrate AEG-1-overexpression.

MP-02. METABOLISM AND ACCUMULATION OF 2-HYDROXYGLUTARATE DURING THE MALIGNANT PROGRESSION OF *IDH*-MUTATED LOW-GRADE GLIOMAS

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OBJECTIVES: Low-grade gliomas WHO grade II (LGG) and secondary glioblastomas WHO grade IV (sGBM) harbor *IDH* mutations in 80%. These mutations lead to accumulation of 2-hydroxyglutarate (2HG). Our objectives were to determine whether accumulation of 2HG in *IDH*-mutated LGG correlates with their malignant transformation and to evaluate changes in the metabolite levels during the malignant progression. METHODS: A total of 54 samples were screened for *IDH* mutations by direct sequencing: 18 pairs of patients with LGG and their consecutive sGBM ($n = 36$) and 18 patients with LGG without malignant transformation. Methanolic extraction of tumor tissue was used for further analysis of the cellular Krebs cycle metabolites: citrate, isocitrate, 2-hydroxyglutarate, α-ketoglutarate, fumarate, and succinate, which were profiled by liquid chromatography-mass spectrometry (LC-MS). Samples from 10 primary GBM and 8 non-glioma tumors were additionally analyzed as a control. To evaluate differences of intracellular 2HG accumulation in both LGG and sGBM groups in comparison to pGBM/non-glioma groups, ratios of isocitrate/2HG were used. The results were correlated with clinical data: time to malignant transformation, progression-free survival, and overall survival. RESULTS: *IDH* mutations were detected in 23/36 patients in both LGG groups (with/without malignant transformation) and were stable during progression to sGBM. Regarding the 2HG accumulation, no statistically significant differences were found between the LGG groups with or without malignant progression. However, as evidence for the validity of our method, significant differences between the LGG/sGBM and the pGBM/non-glioma groups were found. CONCLUSIONS: Intracellular ratios of isocitrate/2HG did not significantly differ between both LGG groups, providing evidence that the level of 2HG accumulation is not a predictable biomarker for distinguishing LGG in relation to their malignant transformation. Furthermore, the isocitrate/2HG ratios remained stable in all pairs of LGG and their consecutive sGBM, assuming that malignant transformation of *IDH*-mutated LGG cells appears to be independent on their intracellular 2HG accumulation.

MP-03. NON-INVASIVE ASSESSMENT OF IDH STATUS IN GLIOBLASTOMA USING DYNAMIC 13C MRS OF HYPERPOLARIZED α-KETOGLUTARATE AND HYPERPOLARIZED PYRUVATE

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INTRODUCTION: Isocitrate dehydrogenase (IDH) mutations have been reported in 70-80% of low-grade gliomas and upgraded glioblastomas (GBM). Because mutant IDH catalyzes α-ketoglutarate (α-KG) reduction to 2-hydroxyglutarate (2HG), IDH status can be assessed by detecting 2HG using 1H magnetic resonance (MR) methods. However, these methods remain challenging. This study evaluates hyperpolarized (HP) αKG and pyruvate as novel translatable MR probes to non-invasively inform on IDH mutational status. METHODS: U87 GBM cells were transduced with viral vectors coding for wild-type (U87IDHwt) or mutant IDH (U87IDHmut). Cells were lysed or grown on microcarrier beads and loaded into a perfusion system. [1-13C]-αKG or [1-13C]-pyruvate were hyperpolarized and rapidly injected into cell lysates (αKG only) or perfused live cells (both compounds); dynamic sets of HP 13C MR spectra were acquired on a 500MHz spectrometer every 3 sec. Gamma-variate analysis was performed on all datasets. RESULTS & DISCUSSION: Injection of HP α-KG resulted in build-up of HP 2HG (183.8 ppm) and glutamate (177.5 ppm) in U87IDHmut lysates, whereas only glutamate was detectable in U87IDHwt. In live cells, HP glutamate produced from α-KG was significantly lower in U87IDHmut cells compared U87IDHwt ($20 \pm 1\%$, $p < 0.05$). HP lactate produced from HP pyruvate was also significantly lower in U87IDHmut cells ($45 \pm 15\%$, $p < 0.05$). Glutamate dehydrogenase and lactate dehydrogenase activities were lower in U87IDHmut by $66 \pm 15\%$ ($p = 0.03$) and $57 \pm 12\%$ ($p = 0.01$) likely explaining the decreased HP glutamate and HP lactate production. Due to the natural abundance [5-13C] α-KG peak at 184ppm, HP 2-HG could not be unequivocally resolved in live cells and alternative approaches are currently being explored. This pioneer study demonstrates for the first time that HP 13C MRS can be used to probe the metabolism of α-KG and that HP α-KG and pyruvate are promising agents for interrogation of IDH mutational status, allowing assessment of metabolic reprogramming associated with the IDH mutation.

MP-04. IRON METABOLISM INFORMS GLIOBLASTOMA STEM CELL MAINTENANCE

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Glioblastoma is a highly lethal cancer that is characterized by florid angiogenesis, invasion into normal brain, and therapeutic resistance. Glioblastomas display cellular hierarchies with a self-renewing, tumorigenic glioblastoma stem cell (GSC) at the apex. The significance of GSCs in tumor biology has been supported by work from our group and others showing that GSCs are relatively resistant to conventional therapies, promote tumor angiogenesis, and are highly invasive. By comparing GSCs to matched differentiated tumor bulk, we have identified a number of molecular targets that function in maintenance of the cellular hierarchy and tumor growth. We have now identified increased iron metabolism as a characteristic of GSCs. Normal glial progenitors accumulate iron to increase migration and proliferation. Brain tumors accumulate iron, and iron regulators are associated with poor survival. As hypoxia induces iron regulators and maintains the cellular hierarchy, we have interrogated iron regulation in GSCs. Ectopic delivery of iron to GSCs increases tumorsphere growth, while disrupting iron metabolism in GSCs using shRNA reduces cell growth and decreases the expression of angiogenic growth factors. Collectively, these results suggest that iron metabolism is important for GSC maintenance and tumorigenic potential.

MP-05. TARGETING TUMOR GLYCOLYSIS AND AUTOPHAGY IN GLIOBLASTOMA *IN VITRO* AND *IN VIVO*

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Using patient-derived glioblastoma xenografts, we have previously shown that the anti-angiogenic drug bevacizumab induces a shift towards a more hypoxic and glycolytic phenotype concomitant with structural vessel normalization and a decreased blood flow within the tumor. We therefore hypothesized that in order to achieve better treatment outcome, anti-angiogenic therapy should be combined with drugs that specifically target survival pathways induced under hypoxia. Here we used cell line and tumor spheroid *in vitro* models to identify promising combinatorial treatment approaches and validated them in patient-derived clinically relevant xenograft models *in vivo*. *In vitro* experiments showed that glioma cell lines were more glycolytic compared to non-tumoral cell lines (astrocytes, endothelial cells), in both normoxic and severe hypoxia as evidenced by the higher expression of glycolysis related enzymes and increased lactate production. Transcriptomic analysis of cultured glioma cells identified genes associated with glycolysis and autophagy to be significantly upregulated in hypoxia. This was confirmed by Western blot analysis showing an increased autophagic flux in glioma cells under hypoxia. To test the effect of anti-glycolytic and anti-autophagic compounds, we utilized 3-dimensional tumor spheroids derived from primary patient-derived glioblastoma as an *in vitro* model system that closely mimics clinical tumor tissue both at the morphological and the genetic level. Clotrimazole, a glycolysis inhibitor, induced cell death in glioma spheroids; however, it was more potent under normoxia compared to hypoxia. Interestingly, in tumor spheroids, chloroquine, an anti-malaria drug and an autophagy inhibitor, had an enhanced cytotoxic effect on spheroids when treated in hypoxia compared to normoxia. Similar results were obtained with mefloquine, another autophagy inhibitor, suggesting autophagy inhibitors as attractive drugs to be combined with anti-angiogenic treatment. Ongoing *in vivo* studies in pre-clinical glioblastoma xenografts will be presented that investigate a possible synergistic effect of bevacizumab with chloroquine.

MP-06. CONSEQUENCES OF IDH1 MUTATION FOR GLIOMA METABOLISM: THERAPEUTIC POSSIBILITIES? AN APPROACH AT THE RNA EXPRESSION LEVEL.

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Mutations of the isocitrate dehydrogenase-1 gene (IDH1) are found in 70% of diffuse infiltrating gliomas. The mutated IDH1 enzyme is located in the cytosol and consumes, rather than produces, NADPH. NADPH levels not only affect cellular proliferation but also mutation rates. Unraveling the effects of the mutated IDH1 on the tricarboxylic acid cycle (TCA) cycle will yield insight in the usage of common metabolic pathways and their alternatives in glial tumors, which could steer new strategies for therapy. In the present study, we used RNA expression profiles of more than 200 diffusely infiltrating gliomas of various malignancy grades. The expression levels of key enzymes involved in glycolysis and in the TCA cycle of gliomas with and without IDH1 mutation, and normal brain samples, were compared. The 10 genes involved in glycolysis and the 23 genes involved in the TCA cycle were investigated. Seven and five genes, respectively, were differentially expressed between the IDH1 mutated and non-mutated samples as compared to normal brain samples. IDH1 mutated cells show several adaptations to maintain sugar metabolism. The expressional differences lead to the recognition of particular metabolic adjusting mechanisms in gliomas with IDH1 mutation. By targeting the differentially expressed enzymes in these gliomas, potential therapeutic targets for interference with the tumor energetic metabolism can be defined.

MP-07. METABOLIC MODULATION OF GLIOBLASTOMA (GBM) BY DEPLETING HEXOKINASE II (HK2): POTENTIATING THE EFFECT OF STANDARD THERAPIES

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We recently demonstrated that hexokinase II (HK2), the first rate-limiting enzyme of glycolysis, is an important mediator of the *Warburg effect* or aerobic glycolysis in glioblastomas (GBMs). Inhibiting HK2 resulted in decreased cell proliferation and increased apoptosis, especially under hypoxia, radiation (RAD), and chemotherapy conditions. Also, depleting HK2 in intracranial GBM xenografts resulted in reduction of tumour growth with enhanced overall survival. Thus, we hypothesize that conditional depletion of HK2 in GBM orthotopic tumours can enhance overall efficacy of RAD and/or temozolomide (TMZ). In order to evaluate the effect of conditional loss of HK2 *in vitro*, we generated GBM cells with conditional expression of HK2shRNA, and these cells were utilized to establish orthotopic mouse xenografts. Bioluminescent imaging (BLI) was utilized to establish the growth kinetics of GBM tumors intracranially. *In vitro* results showed that conditional loss of HK2 sensitizes GBM cells to TMZ and TMZ/RAD, with decreased cell proliferation and increased DNA damage, and this appears to be hypoxia dependent. The synergistic effect of loss of HK2 along with RAD and/or TMZ is evaluated *in vivo* to determine whether HK2 status influences the chemo- and radio-sensitivity of GBMs. Conditional loss of HK2 significantly improved the survival of glioma-bearing mice, and the survival was further enhanced in RAD group and combined RAD/TMZ group. We anticipate that targeting key metabolic enzymes involved in modulating the *Warburg effect*, such as HK2, may provide a novel therapeutic approach in combination with other classes of anti-cancer drugs and will be instrumental in managing GBM patients.

MP-08. SILENCING MCT4 INHIBITS GBM NEUROSPHERE GROWTH IN A LACTATE EXPORT-INDEPENDENT FASHION

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Glioblastomas (GBMs) contain a hypoxic core surrounded by proliferative cells. To unmask genes important in hypoxia, we exposed 2 GBM neurosphere lines, HSR-GBM1 and JHH-GBM10, to 1% and 21% oxygen levels for 24 hours and compared gene expression. We identified SLC16A3 (monocarboxylate transporter-4 [MCT4]) as one of the most upregulated genes in response to hypoxia. To investigate the clinical importance of MCT4 in GBM, we examined the Kaplan-Meier survival curves of glioma patients using public databases. We found that patients with at least two-fold upregulation of MCT4 have a significantly shorter survival ($p < 0.0001$) than patients with intermediate expression. Consistent with this, MCT4 upregulation correlated with the aggressive mesenchymal subset of GBM ($p < 0.0001$) and MCT4 downregulation correlated with the less aggressive CIMP (CpG island methylator phenotype) subset of GBM ($p < 0.0001$). We next examined MCT4 protein levels using immunohistochemical analysis of tissue microarrays, confirming that MCT4 protein levels were increased in high-grade as compared to lower-grade astrocytomas ($p < 0.0001$), and further suggesting that MCT4 is a clinically relevant target. To test the requirement for MCT4 in vitro, we transduced neurospheres with lentiviruses encoding short hairpin RNA against MCT4. We found that cell growth was inhibited in hypoxia in both neurosphere lines. Interestingly, while MCT4 was expressed at lower levels in normoxia, silencing in 21% oxygen also significantly slowed growth. This slowed growth was associated with reduced clonogenicity and increased apoptotic fraction in HSR-GBM1. Importantly, MCT4 silencing also slowed GBM intracranial xenograft growth in vivo ($p = 0.009$). Interestingly, while MCT4 is a well-characterized lactate exporter, we found that extracellular lactate levels did not change following MCT4 silencing, suggesting a novel lactate export-independent mechanism for growth inhibition in malignant gliomas.

MP-09. UNDER CONDITIONS OF NUTRIENT DEPRIVATION, LYN FACILITATES GLIOBLASTOMA CELL SURVIVAL BY PROMOTING AUTOPHAGY AND INHIBITING CELL DEATH

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Members of the Src family of kinases (SFKs) can promote cell survival under nutrient-rich conditions by inhibiting apoptosis. Here, we examined the role of Lyn, a SFK, in promoting glioblastoma (GBM) tumor cell survival under nutrient-rich and nutrient-deprived conditions, based on our prior work showing that Lyn activity and protein are significantly elevated in GBM biopsies. Under nutrient-rich conditions, lentiviral-mediated expression of constitutively-active Lyn (CA-Lyn) or dominant-negative Lyn (DN-Lyn) in GBM cells had no effect on cell survival as compared to cells transduced with the vector alone (LV). In the absence of supplementation with L-glutamine and FBS, however, CA-Lyn expression promoted autophagy and inhibited cell death, and DN-Lyn expression inhibited autophagy and promoted cell death. Activation of AMPK has been shown to promote autophagy and result in reduced pS6 kinase. In the nutrient-deprived cells, CA-Lyn expression enhanced AMPK activity and reduced the levels of pS6 kinase, and DN-Lyn reduced AMPK activity and enhanced the levels of

pS6 kinase. Similar results were obtained using another cultured GBM cell line, primary glioma stem cells, and when GBM cells were propagated in the brains of nude mice. Our findings suggest that in nutrient deprivation, which occurs in tumors *in vivo*, Lyn acts to enhance the survival of GBM cells by promoting autophagy and inhibiting cell death.

MP-10. METABOLIC PROFILES OF KETOLYSIS AND GLYCOLYSIS IN MALIGNANT GLIOMAS: POSSIBLE PREDICTORS OF RESPONSE TO KETOGENIC DIET THERAPY

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Warburg showed that malignant tumors are highly dependent on glucose metabolism. Furthermore, some tumors may be deficient in enzymes needed to metabolize ketones. These enzymatic differences in energy metabolism between normal brain tissues and malignant gliomas formed the basis for animal model studies that showed increased survival in mice with orthotopically transplanted glioblastoma multiforme (GBM) and treated with a calorie-restricted ketogenic diet (CRKD). To test the hypothesis that human brain tumors may also be sensitive to CRKD, we used immunohistochemistry reactions on formalin-fixed, paraffin-embedded tumor samples to evaluate for the presence of enzymes important for the metabolism of ketones and glucose. Immunoreactivities were graded using a semiquantitative scale based on the percentage of positive cells: negative $<5\%$ (NEG); intermediate 5-20% (INT); and positive more than 20% (POS). Focal non-neoplastic "normal" brain tissue present within the specimens expressed the enzymes and served as a positive internal control. Succinyl CoA: 3-oxoacid CoA transferase (OXCT1) and 3-hydroxybutyrate dehydrogenase 1 (BDH1) are mitochondrial enzymes important for metabolizing beta hydroxy buterate, the main ketone in blood. Both of these enzymes were either decreased or absent (INT or NEG) concordantly in 14 of the 17 (82%) GBM specimens and in 1 of the 6 (17%) anaplastic astrocytomas (AA). Two of the enzymes in the glycolytic pathway hexokinase-2 and pyruvate kinase M2 were concordantly NEG or INT in only 3 of the 17 GBMs that also were NEG or INT for both OXCT1 and BDH1. The remaining brain tumors were positive for at least one of these glycolytic enzymes. To show that mitochondrial enzymes were not globally deficient, the tissues were evaluated for the mitochondrial enzyme acetyl CoA transferase (ACAT1). Of the 14 GBM specimens that had NEG or INT mitochondrial enzymes OXCT1 and BDH1 only 4 were INT and 1 was NEG for ACAT1. The cytoplasmic enzyme BDH2 was INT or NEG in only 4 of the GBMs and was POS in all 6 of the AA specimens. In conclusion these data showing that many, but not all, malignant gliomas lack ketolytic enzymes support the rationale of a CRKD as investigational treatment for patients with maliganant gliomas. We hypothesize that patients with diminished capacity to metabolize ketones likely may derive the most benefit from treatment with CRKD.