Lactate/Creatine Ratio Tracks with HIF-1 Alpha Accumulation in High-Grade Astrocytoma Cells

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Introduction: High-grade astrocytoma contain hypoxic sub-regions that are, paradoxically, highly vascularized. It is thought that hypoxia may lead to an accumulation of HIF-1α that activates genes that play a role in angiogenesis, metabolism and oxygen transport. In normoxic conditions, HIF-1α is continuously degraded via proteosomal processes[1]. Exposure to hypoxia or transition metals such as cobalt oppose the degradation processes, thereby allowing HIF-1α to accumulate in cells[1]. Hypoxic tumor is known to be resistant to standard radiotherapeutic strategies and HIF-1α mediated mechanisms likely contribute to the radioresistance. The ability to identify hypoxic regions with neuroimaging methods such as magnetic resonance spectroscopy (MRS) would enable such regions to be treated with additional focal therapy. A common feature of MR spectra from Grade IV astrocytoma (GBM) is an elevation in the lactate peak[2], which has been postulated to result from anaerobic metabolism in hypoxic tumor sub-regions. However, no studies have confirmed the link between MRS-visible lactate and hypoxia in GBM. Our experiments involved two high-grade astrocytoma cell lines that differ in the expression of Akt, a mediator of HIF-1α expression, that have previously been shown to form intracranial tumors in rats that resemble human Grade III (Akt-)[3]and Grade IV (Akt+)[4] astrocytoma. The tumors grow at a similar rate for the first 20-24 days, after which the Akt+ tumors grow at an exponentially faster rate presumably due to the difference in the response to hypoxia[4].We investigated the role of Akt and cobalt stabilization in the accumulation of HIF-1α and the association between HIF-1α and lactate/creatine ratios within the cells.

Methods: *Immunofluorescence* Cells were maintained in DME H-21 with 10% FBS and 1% penicillin/streptomycin at 37°C. In order to stabilize HIF-1α in a manner similar to hypoxia, 0.1 mM cobalt chloride ($CoCl_2$) was added to one set of each cell type four hours before fixing. Cells were labeled with a primary mouse anti-HIF-1α antibody (Novus, Littleton, CO), a biotinylated secondary antibody (Vector, Burlingame, CA), and the fluorophore AF555 (Invitrogen, Carlsbad, CA). Slides were mounted with mounting media that contained a DAPI counterstain and imaged with a fluorescence microscope (Zeiss, Germany). Image Pro Plus (Media Cybernetics, Silver Spring, MD) was used to calculate the ratio of HIF-1α-positive (red) cells to the total number of DAPI-positive (blue) cells. A two-tailed t-test was performed with an alpha-level of $p<0.05$ as a threshold for significance. *HRMAS MRS* Cells were grown in normal or $CoCl_2$ media (4 hours) in T150 flasks. They were washed in a D_2O-based PBS buffer and transferred to HRMAS rotor conformant capillary tubes. Samples were slow cooled (1° C per minute) to –80°C by controlled freezing. HRMAS MR spectra were acquired using a Varian Inova 500 Mhz spectrometer equipped with a gHX gradient nanoprobe, at 1°C. Acquisitions were obtained at a 2250 Hz spin rate using the standard one pulse proton sequence with water saturation. Metabolite peak areas were integrated using the NMR analysis software package ACDlabs (Advanced Chemistry Development, Ontario, Canada). The levels of the following metabolites were measured: glutathione+glycine+cysteine (Glx, 3.77-3.79 ppm), glycerophosphocholine+phosphocholine (GPC+PC 3.22-3.25 ppm), creatine+phosphocreatine (Cre, 3.02-3.04 ppm), and lactate (Lac, 1.32-1.35 ppm). Metabolite levels were reported as ratios to the Cre peak. A two-tailed ANOVA ($p < 0.01$) was used to compare the level of each metabolite in the two cell lines with and without $CoCl_2$.

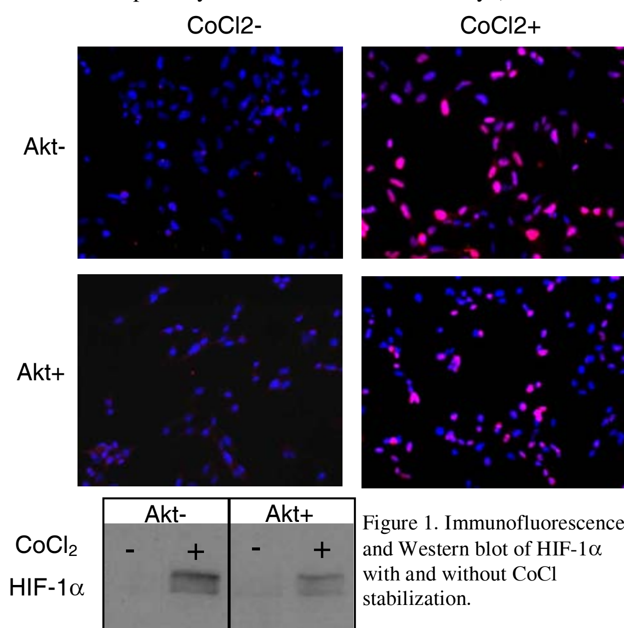

Figure 1. Immunofluorescence and Western blot of HIF-1α with and without CoCl stabilization.

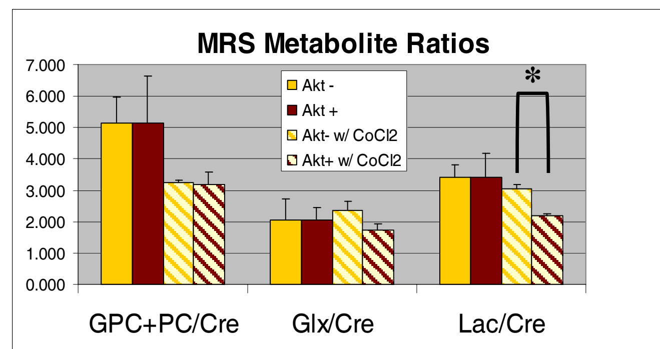

Figure 2. HRMAS MRS metabolite ratios

Results: Only cells that were exposed to $CoCl_2$ showed specific staining indicative of the presence of HIF-1α (Fig 1). Surprisingly, higher HIF-1α levels were consistently observed in Akt- cells (0.46 +/- 0.11) compared to Akt+ cells (0.19 +/- 0.12) (N=10, p=0.0004). Consistent with the HIF-1α level, no differences were observed between any of the MRS peak ratios from the two cell lines under normal growth conditions (N=3 each cell line); however, $CoCl_2$ stabilization of HIF-1α resulted in higher Lac/Cre in the Akt- than the Akt+ cells (3.04 +/- 0.14 vs 2.19 +/- 0.07, N=3, p=0.0007) (Fig 2).

Discussion: These results suggest a biological relationship between the MRS Lac/Cre ratio and HIF-1a status in astrocytoma. The unexpected decrease in Lac/Cre ratio in both cell lines after exposure to $CoCl_2$ suggests that Cre rather than Lac may be more closely associated with HIF-1a levels in the cells. *In vivo* and *ex vivo* MRS studies of tumors grown from these cells are needed to determine whether Lac/Cre may be used as a surrogate clinical marker for HIF-1a status.

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