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Qualitatively Assessing the Expression of Kynurenine Pathway Metabolic Enzymes in Human Glioma Tissue

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Abstract

Gliomas are the most common brain tumor and glioblastoma multiforme (GBM) is the most common type of glioma observed in patients. Prognosis with late stage glioma and GBM is very poor even with recent advances in treatment. Along with treating the tumor, manners of bypassing the blood brain barrier and ensuring the tumor uptakes the treatment is necessary. Because the treatments can harm regular brain cells there is concern about the viability and toxic side effects of many treatments. With all of these challenges posed it is important to understand how glial tumors can be so recurring and difficult to treat. One method employed by the tumors is utilizing the kynurenine pathway of tryptophan metabolism to suppress the immune system and promote tumor proliferation. Tryptophan is brought in to the cell by L-amino acid transporter (LAT1) and catabolized in to tryptophan by indoleamine 2,3 dioxygenase I/II (IDO-1/IDO-2), tryptophan 2,3 dioxygenase (TDO-2), kynurenine-3-monoxygenase (KMO), and kynureninase (KYNU). Finally this kynurenine can interact with the aryl hydrocarbon receptor (AHR) to promote gene transcription that allows the tumor to further thrive. Immunohistochemical staining across all grades and types of gliomas reveals expression patterns for the enzymes involved in the tryptophan metabolism. The staining revealed high, near-ubiquitous expression for IDO-2, TDO-2, KYNU, and LAT-1. KMO and IDO-1 had staining in the majority of the tissues and AHR showed the least expression. A repeat of this experiment with more cases is required so that a statistical analysis may be done on the expression patterns for the proteins. The six proteins with high expression show promise as viable targets for immunotherapy but more *in vitro* and *in vivo*

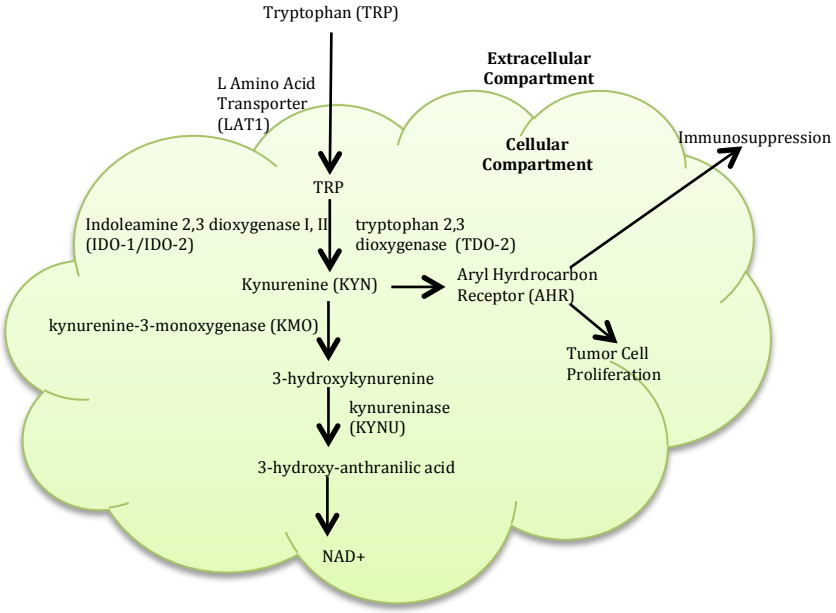
experiments are required to determine if they are feasible treatments.

Introduction

Overview of Glioma:

Gliomas comprise a heterogeneous group of neuroectodermal tumors with distinct clinical, histological, and molecular characteristics that arise from the glia, the supporting cells of the CNS. The World Health Organization (WHO) classification classifies gliomas by predominant cell type and grade (I to IV). The histologic grading of gliomas takes into account nuclear atypia, cellularity, mitotic activity, endothelial proliferation, necrosis, and proliferation index. Glioblastoma multiforme (GBM) is the most common and most aggressive type of glioma with over 25,000 new cases diagnosed annually in the USA. It is highly invasive and its high chance of recurrence along with heterogeneity makes GBM very difficult to treat. We have previously shown that gliomas use tryptophan metabolism to increase immunosuppression and cell proliferation (Juhász *et al.*, 2006). The mechanism by which the tumors accomplish this is the kynurenine pathway of tryptophan metabolism. This is a regularly occurring pathway in human metabolism, but the tumor cells utilize it as a mechanism to become more invasive.

It begins with tryptophan being transported into the cell by the L-amino acid transporter (LAT1). Then tryptophan is catabolized into kynurenine by indoleamine 2,3 dioxygenase-1 (IDO-1), indoleamine 2,3 dioxygenase-2 (IDO-2) and tryptophan 2,3 dioxygenase (TDO-2) (Bakmiwewa *et al.,* 2012; Platten *et al.,* 2012; Adams *et al.,* 2014). This kynurenine is then catabolized into 3-hydroxykynurenine by kynurenine-3-monoxygenase (KMO). Kynureninase (KYNU) then converts 3-



hydroxykynurenine to 3-hydroxy-anthranilic acid (Adams *et al.,* 2014). The final product is NAD+, which is a compound required by the body for cellular respiration, energy production, and DNA repair. The kynurenine produced by this catabolism can promote immunosuppression and cell proliferation in conjunction with the transcription factor aryl hydrocarbon receptor (AHR) (Adams *et al.,* 2012). The tryptophan metabolite kynurenine suppresses antitumor immune responses and promotes tumor-cell surivival and motility through the AHR in an autocrine/paracrine fashion resulting in gene transcription (Opitz *et al.,* 2011). Other ways in which the tryptophan metabolism can promote tumor proliferation is through a shock from tryptophan depletion where lymphocytes will have arrested growth at the G1 phase (Munn *et al.,* 1999*)*. Derivatives of the kynurenine pathway such as 3-hydroxyanthranlic and quinolinic acid can induce T-cell apoptosis (Fallarino *et al.,* 2002) and can also cause the differentiation of T-cells (Fallarino *et al.,* 2006). In astrocytic tumors low numbers of T-cells and T-cell toxicity were observed along with defects in the function of these T-cells (Dix *et al.,* 1999).

<u>Review of Treatment:</u>

For the last 10 years, the standard therapy has remained maximal safe surgical resection followed by external beam radiation with concomitant administration of temozolomide (TMZ), followed by monthly TMZ (referred to as the Stupp regimen) (Wait *et al.,* 2014). Even with this standard treatment regimen the standard 2-year survival rate is just 25% of patients (Tellingen *et al.,* 2015). Various factors contribute to the difficulty of treating GBM including their heterogeneity, which results in non-identical genetic makeup between different cell types making genetic targeting more difficult. The blood brain barrier prevents many treatments from reaching the site of the tumor because it prevents particles over 500 Da from passing through. This means only a select few types of small, lipophilic molecules can get past the barrier. This presents a major obstacle in the delivery of chemotherapy (Okongi *et al.,* 2015). To bypass this, carmustine wafers are widely used as a form of local chemotherapy delivery. The medication is placed in the resection cavity resulting form surgery so that the chemotherapy will have direct contact with tumor cells. This treatment in combination with temozolomide showed marked increase in patients' 1-year and 2-year survival rates, going from 69% to 81%, and 29% to 47% respectively (Affronti *et al.,* 2009). This direct of a treatment presents issues because as with all chemotherapy there is a chance for detrimental and toxic side effects. Carmustine wafers were shown to produce grade 3 or 4 adverse events in 19 people in a 44-person study (43%) (Bock *et al.,* 2010). Along with these stem-like glioblastoma cells demonstrated resistance to temozolomide via MEK-ERK induced activation of O(6)-methylguanine DNA methyltransferase (MGMT). This enzyme is utilized by tumor cells to demethylate

the temozolomide and reduce its effectiveness. (Sato *et al.,* 2011). In the face of these difficulties with treatments, different methods involving immunotherapy have been created to try and target specific metabolic, signaling, and developmental pathways in tumor cells. One of the methods that has shown some promise is the use of a cancer vaccine where patients are vaccinated with their own dendritic cells. These cells would be programmed to combat tumor growth and cancer stem cells. For patients with malignant gliomas, treatment with the vaccines showed an increase in median survival time and five-year survival in newly diagnosed patients (Chang *et al.,* 2011). Inhibition therapy is the one of the most researched methods of treatment for gliomas, with the basis of targeting certain genetic markers or proteins necessary for tumor growth. Epidermal growth factor receptor (EGFR) has been shown to be a major factor in tumor growth and proliferation (Jorissen *et al.,* 2003). EGFR has been the target of inhibition therapy with the use of small tyrosine kinase inhibitors (TKI) but a study by Mellinghoff *et al., 2015* proved that the inhibition treatment in addition to radiotherapy was not effective. Similarly Vascular endothelial growth factor (VEGF) was identified as a target and the drug bevacizumab (a monoclonal antibody targeting VEGF) showed potential as a possible adjunct treatment. An institutional randomized phase III trial was performed with 978 patients and although the group with bevacizumab treatment showed a longer progression free survival (10.7 months compared to 7.3 months) there were also higher rates of neurocognitive decline, increased symptom severity, and a decline in health-related quality of life (Chinot *et al.,* 2011).

Inhibition of Parts of Kynurenine Pathway:

Inhibition of IDO-1, IDO-2, or TDO-2 could be effective treatments but because of their redundancy targeting these enzymes can be difficult. IDO-1 seems to have an intricate link to oncogenic signaling pathways, and it seems that inhibition of TDO-2 can complement inhibiton of IDO-1 (Platten *et al.,* 2015). It has been proven *in vivo* that IDO inhibition can produce regression of tumor growth in different experiments. In one experiment focusing on breast cancer a group used 1-methyl-DL-tryptophan (1MT) in combination with other cytotoxic agents and saw a 30% decrease in tumor volume within two weeks of delivery as compared to very limited inhibition of growth by a standard chemotherapeutic treatment (Muller *et al.,* 2005). While inhibiting IDO-1 or IDO-2 seems viable, the role of TDO-2 must be considered because of its redundancy and similar function to IDO-1 and IDO-2. The effects of inhibiting TDO-2 have been examined and multiple compounds have shown to be viable inhibitors. TDO-2 has been tied to the FKBP52 gene and it was shown that by regulating the expression of FKBP52 that TDO-2 expression could be constrained and this could be beneficial to patients (Ott *et al.,* 2014) In a study of TDO-2 inhibitors it was shown that certain inhibitors such as compound LM10, which inhibits TDO-2 and not IDO-1 or IDO-2. By targeting TDO-2, IDO-1, and IDO-2 it makes treating a larger percentage of tumors more viable. By targeting both 51% of tumors could be treated (Pilotte *et al.,* 2012). Other downstream enzymes such as AHR, KMO, and KYNU present targets for inhibition and treatment. AHR inhibition has proven to reduce glioma invasiveness, clonogenicity and proliferation via the AHR antagonist CH-223191 or gene silencing (Gramatzki *et al.,* 2009). Both KYNU

and KMO have had inhibitors developed to examine their effect on other conditions but there has not been any extensive study on their effects in brain tumors. Caution must be taken with inhibition treatment because blocking parts of such vital pathways can cause immune system over activation resulting in unresponsiveness (Gostner *et al.,* 2015). Standard treatment for glial tumors does not produce the most promising prognosis so looking for new ways to treat the cancer is necessary especially with the difficulties dealing with drug and radiation resistant tumors. Immunotherapy in brain tumors is being constantly furthered and more research about possible treatment targets is necessary to achieve better patient outcomes. Our study will qualitatively examine the expression of LAT1, IDO-1, IDO-2, TDO-2, AHR, KMO, and KYNU across the various grades of glioma through the use of immunohistochemistry in order to gain a basic understanding about the viability of these compounds as targets for treatment.

Methods and Materials

Immunohistochemistry:

Resected glioma tissues were collected from the operating room at the time of surgery. A portion of the sample was fixed in formalin and then embedded in paraffin. The tissue is sectioned on to a charged slide and then a staining procedure is performed. The protocol begins with de-paraffinizing the slides with two five-minute xylene washes followed by a series of three-minute ethanol washes in 100%, 95%, and 70%. After de-paraffinization the sections are exposed to antigen retrieval with a VECTOR antigen demasking solution (prepared with 940 µL of stock solution in 100 mL of deionized water) while at high heat. The tissue is then rinsed

in Phosphate buffered saline (PBS) for five minutes, and phosphate buffered saline with triton (PBST) for five minutes. The peroxidase activity is quenched with a 0.3% hydrogen peroxide blocking solution for 30 minutes. After a five-minute wash in PBST, VECTASTAIN anti-rabbit and anti-mouse kits are used. A goat serum for anti-rabbit antibodies and a horse serum for anti-mouse antibodies is diluted in PBST is applied to block any non-specific antibody binding (20 minutes of blocking), and then primary antibodies (stock primaries diluted in blocking serum) targeted to bind to TDO-2, IDO-1, IDO-2, AHR, LAT1, KMO, and KYNU are applied for one hour. TDO-2, IDO-1, AHR, LAT1, KMO, and KYNU are diluted to a 1:100 concentration whereas IDO-2 is diluted to 1:50. After two five-minute PBST washes a secondary antibody (50μL in 10 mL of blocking solution) is applied that recognizes and binds to the primary antibody. Both antibodies are grown in the same host species (rabbit or mouse). Afterwards VECTASTAIN ABC reagent is used to allow the tissue to react with the DAB staining substrate. The DAB substrate interacts with the secondary antibody and causes a color stain to appear indicating presence of any of the target compounds. A hemotoxilin counterstain is performed and slides are rinsed with ethanol and xylene for 10 seconds at a time before being mounted with a cover slip. Later the slides are imaged using an Olympus camera microscope. These studies were approved by the Wayne State University Institutional Review Board and written informed consent was obtained from all participants.

Antibodies used

AHR using GeneTex GTX22765 anti-AHR antibody, IDO-1 using Novus NBP-1-87702 anti-IDO-1 antibody, IDO-2 using Aviva OAAB08672 anti-IDO-2 antibody,

TDO-2 using Novus NBP-2-13424 anti-TDO-2 antibody, KYNU using Novus NBP-1-56545 anti-KYNU antibody, KMO using Proteintech 60029-1-1G anti-KMO antibody, and LAT1 using Abcam ABS5226 anti-LAT1 antibody.

Results

Expression of Kynurenine Pathway Enzymes:

Immunohistochemical staining was performed on samples of various grades of glioma for TDO-2, IDO-1, IDO-2, KYNU, LAT1, KMO, and AHR. Upon examination of the stains we observed to see if there was a correlation between the increasing grades or the composition of the tumor and the intensity of expression and no clear correlation was found. The number of samples used was too low to provide statistically relevant correlation but the qualitative assessment shows ubiquitous expression of IDO-2, TDO-2, KYNU, and LAT1 across all types of gliomas. IDO-1 and KMO have similar expression patterns with less consistent expression than IDO-2, TDO-2, KYNU, and LAT1 but still showing expression in the majority of tissues. AHR was the least expressed in the different grades with the exception of the GIII Oligoastrocytoma. As this was simply a qualitative assessment more samples and cases would need to be examined in order to obtain more statistically relevant results. These results were determined by comparing the staining to that of the no primary controls seen in the figures.