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Targeting Galectin-1 as a Potential Therapeutic for Glioblastoma

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by

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ABSTRACT

Wayne Luther Glore. Targeting Galectin-1 as a Potential Therapeutic for Glioblastoma (Under guidance from Arabinda Das & Michael Ostrowski)

Glioblastoma (GB) is classified by the World Health Organization (WHO) as a Grade IV astrocytoma characterized by a poor prognosis with a median survival time ranging from 15-16 months. The standard of care for GB is surgery followed by radiation and chemotherapy treatment with Temozolomide, but even with the aggressive treatment, GB recurrence occurs in approximately 90 % of the patient population. New treatment options have been FDA approved which include Novocure's Optune Device and Genentech's Avastin, but neither of these options drastically change survival time or quality of life. Galectin-1 (Gal-1), a protein with a high affinity to bind β-galactosides, has been implicated in other cancers such as renal cancer, liver cancer, and urothelial cancer and plays a role ranging from angiogenesis to altering the tumor microenvironment for immune suppression. Little research has been conducted investigating Gal-1's role in GB so the aim of our in vitro and in vivo studies was to gain a better understanding of Gal-1's potential mechanisms in GB and see the feasibility of Gal-1 inhibition as a potential treatment option. The data collected illustrated roles of Gal-1 in angiogenesis, in apoptosis, and in facilitation of a hypoxic environment. Inhibition of Gal-1 shows signs of being a plausible treatment option especially if given coadjuvant to an Anti-VEGF therapy.

Acknowledgements

I dedicate this page to those who have helped me along the way. I want to thank my parents Mark and Suzanne Glore for illustrating the importance of continuing education and having a mindset of determination. I send my gratitude to my mentor Arabinda Das who has greatly enhanced my learning of experimental design and what types of questions to ask to dive deeper into neuro-oncology research and research in general. He has provided me insightfulness into many different molecular biology techniques I have not known previously. I thank my co-mentor Michael Ostrowski, and committee members David Cachia, Mark Rubinstein for asking questions to guide me in the type of knowledge and concepts I should be prioritizing in the pursuit of knowledge and research questions ask. Last of all, I want to thank Medical University of South Carolina for the last great two years I have had learning and soaking in all the knowledge I could.

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List of Abbreviations

BBB- Blood Brain Barrier

EGFR- Epidermal Growth Factor

FGFR 4- Fibroblast Growth Factor Receptor 4

Gal-1 – Galectin-1

GB- Glioblastoma

IDH 1- Isocitrate Dehydrogenase 1

IDH 2- Isocitrate Dehydrogenase 2

MAP 3K1-Mitogen Activated Protein Kinase Kinase Kinase 1

MGMT- O^6-methylguanine DNA Methyltransferase

NF 1- Neurofibromatosis Type 1

PTEN- Phosphatase and Tensin Homolog

VEGF- Vascular Endothelial Growth Factor

Tam- Tumor Associated Macrophage

TET 1- Ten-eleven Translocation Methylcytosine Dioxygenase **1**

TP 53-Tumor Protein 53

TME- Tumor Microenvironment

Treg- Regulatory T Cell

WHO- World Health Organization

Chapter 1

Introduction

Glioblastoma (GB) is categorized by the World Health Organization (WHO) as a grade IV astrocytic tumor and stems from astrocytes, a star shape glia cell that protects the brain from infections and diseases. The exact cause for the onset of the brain tumor is unknown but begins when astrocytes start to rapidly proliferate to form tumors due to variations in genetic expression that promote cell growth. GB can cause a multitude of symptoms that include invasion and decay of healthy brain tissue surrounding the tumor, pressure from fluids building up, and a disruption of cerebrospinal fluid circulation through the brain. The tumor is typically found in the cerebral hemispheres of the brain, but it has the capacity to occur at any location in the brain. It is a cancer that is plagued with low survival rates with the median survival rate at 12-18 months. The disease's 5-year survival rate is approximately 10%. There are many barriers that prevent development of effective treatment for GB.

Barriers to Treatment

Multiple barriers thwart effective treatment of GB. First, GB has high heterogeneity regarding the genetic composition of the tumor. GB cells capitulate multiple neurodevelopmental and

lineage differentiation processes to become their specified neuronal cells in the brain.[1] This differentiation of cells leads to the genetic diversity that encompasses GB. Second, genetic expression profile differs between patients with GB. No one or two genes are the primary genetic drivers for tumor growth and eventual GB. A list of several indicated genes for GB progression and sustained growth include TP 53, PTEN, EGFR, IDH 1, IDH 2, MAP 3K1, NF 1, TET 1, and FGFR4. Due to the multitude of different genes expressed and their mutations in GB, it is difficult to target a single gene that will have beneficial treatment for a vast subset of patients. Even if one specific gene became readily targetable the treatment would still face difficulty in delivery to the tumor because of the blood brain barrier (BBB).

Advances in GB treatment prove futile due to the BBB, a defense system that limits the entry of molecules and blocks the entrance of toxins or infectious molecules into the brain. The BBB is the barrier between the cerebral capillary blood and the interstitial fluid of the brain consisting of capillary endothelial cells, basement membrane, neuroglial membrane, and glial podocytes, i.e., projections of astrocytes.[2] Tight junctions are formed between the endothelial cells preventing entry from blood borne pathogens and other potential deleterious products.

Only certain products can bypass the blood brain barrier these include lipid soluble molecules, and receptor-mediated transport of glucose and ions to pass through. Drugs given by intravenous (IV) injection and subcutaneous injection have low probability of crossing the BBB and being delivered to the target site of the tumor. The inability to easily cross the BBB with innovative treatment ideas is a powerful barrier to new treatment methods being developed and eventually FDA approved.

Recurrent Glioblastoma Progression

Recurrent GB occurs in majority of patients with initial GB. The difficulty resides in removing the whole tumor and infiltrating tissue around the tumor and complete resection is uncommon. No established standard of care has been established for recurrent GB and treatment generally consist of another round of tumor re-section followed by radiation and treatment with Temozolomide. Survival time is relatively low and still rest at a little over a year. Recurrent GB is less responsive to treatment and has a high rate of drug and radiation resistance.

Current Standard of Care

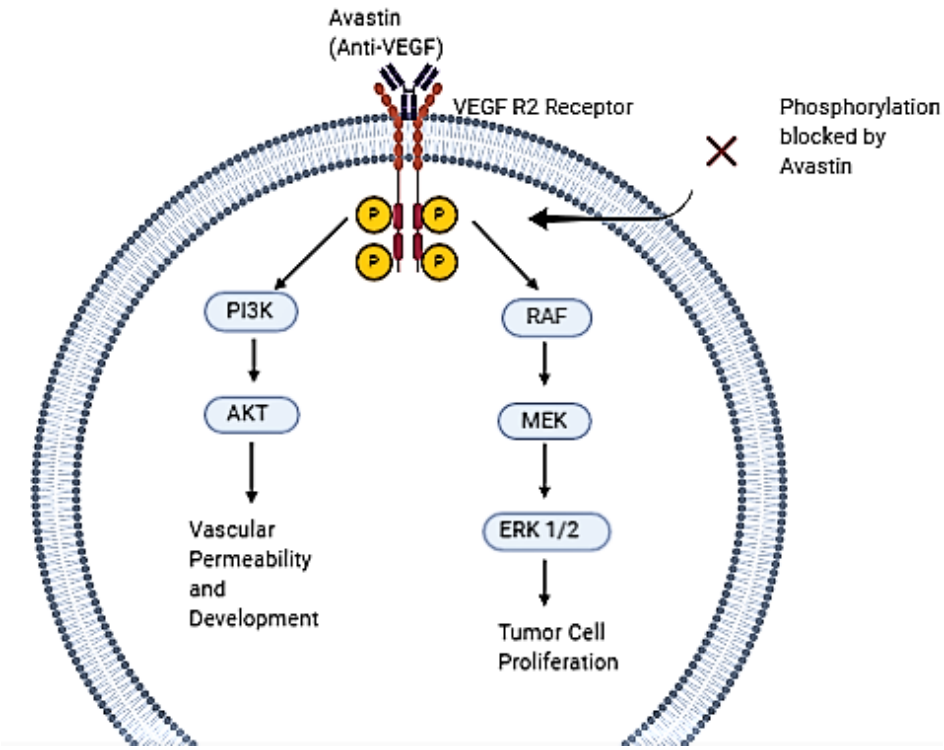
The standard of care for initial GB is maximal surgical resection followed by radiotherapy then Temozolomide (TMZ). A new treatment Bevacizumab (Avastin) was investigated

specifically for recurrent GB, but overall survival (OS) was not increased only quality of life (QOL). The benefits of TMZ + Bevacizumab help with increased time of progression free survival. The current standard of care was established in 2005 with maximal safe resection and then concomitant daily temozolomide and radiotherapy followed by adjuvant temozolomide showed improvement in median OS and 2-year survival.[3] TMZ is a chemotherapy treatment that is an alkylating agent that binds to DNA in cancer cells preventing their division and growth. Clinicians and advancing research identified the importance of DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) in understanding the likeliness of tumor response to TMZ treatment. MGMT would repair the damaged DNA caused by TMZ but in cases where the MGMT is methylated it loses function and eventually degrades. Lack of methylation of the MGMT gene is associated with a stronger likeliness of TMZ resistance by the tumor in patients who have GB. [4-7]

Avastin: Potential Implications GB and Recurrent GB Treatment

Bevacizumab, also known as Avastin, is a drug which is made by the company Genentech. Bevacizumab is a humanized monoclonal Anti- Vascular Endothelial Growth Factor (VEGF) antibody that

blocks all three isoforms of VEGF-A and blocks interactions with the VEGF R2 receptor. VEGF expression in GB and recurrent GB is associated with vascular permeability, tumor cell proliferation, and tumor cell migration.



Schematic 1.1: *Blockage of VEGF Signaling Cascades by Avastin* (*Made through Biorender)*

Schematic 1.1 illustrates the pathways that become active, both PI3K/AKT and RAF/MEK/ERK1/2, to promote vascular development for the tumor and increase proliferation of tumor cells. Ligand VEGF-A, the ligand that typically binds to the VEGF R2 receptor, has other roles involved in activating migration and

proliferation of endothelial cells (ECs) to maximize angiogenic capacity of the tumor microenvironment. Abundance of VEGF-A growth factor creates a gradient of soluble chemo attractants for endothelial cells to migrate towards.[8] VEGF inhibition has the potential to be moderately dangerous to certain patient subsets, and recent evidence demonstrates that Anti-VEGF mAb inhibition leads to problems such as nephrotoxicity and can be deadly to patients.[9] VEGF is an essential growth factor to promote angiogenesis and healthy functioning of blood vessels. Thus, there is a necessity to look at other forms or methods to target cancer's highly immunosuppressive immune environment.

Chapter 2: Review of the Literature

This chapter will discuss the current literature in the field of Glioblastoma and general cancer research by examining mechanisms of Recurrent Glioblastoma, current trend in cancer therapeutics, Galectin-1 in cancer progression and immunosuppression, the role of both hypoxia and radiation in the TME and describe why our therapeutic approach is innovative.

Mechanisms of Recurrent Glioblastoma

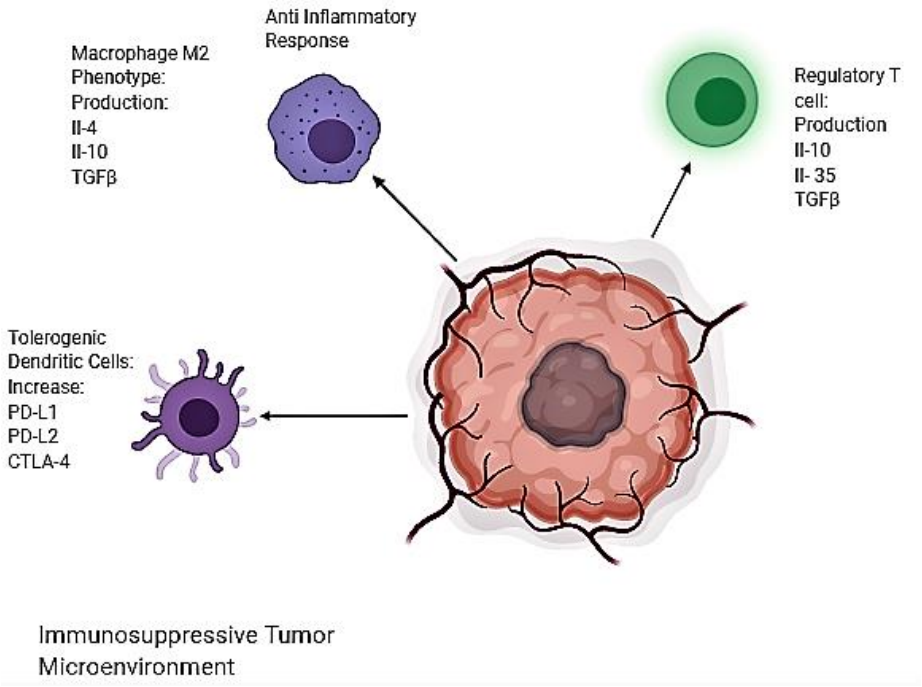
Many different cellular and extracellular mechanisms play a part in establishing resistance to treatment for recurrent GB. Cancer stem cells (CSCs) in the tumor have properties that lead to

adaptation and response to the microenvironment and treatment methods. CSCs are referred to as tumor initiating cells due to their high capacity to generate tumors in xenograft models.[10-11] CSCs for GB are derived from neural stem cells and are believed to be the driving factors that lead to the genetic diversity encompassing the tumor. Cancer researchers have a consensus that cancer stem cells are what drives resistance and recurrence. The common marker for CSCs is CD 133.[12] Recurrent Glioblastoma is challenging to make effective therapeutics for due to its strong drug-resistance through hypoxia and vast expression of CSCs, immunosuppression, and similarities to tissue necrosis due to irradiation.

Recurrent GB cells gain a further capability for drug suppression. Initial GB naturally has small amplitude of drug suppression capacity due to inefficiency of drug deliver. The previously discussed blood brain barrier limits the quantity of treatment that gets to the targeted tumor in the brain. There is no efficient way of direct targeting removal of the entire tumor due to the stress that additional surgery will have on the brain and the potential disruption of the glymphatic system. Recent evidence illustrates that there is a small set of cancer cells within the tumor that are named persisters due to their relative similarity to colonies in E.coli culture that are persistent to antibiotic treatment.[13] These cancer persister cells

gain drug-tolerant capabilities through mechanisms of either chromatin or metabolic remodeling.[13] Evidence has shown that treatment resistance for GB can derive from gene mutations and slow growing cancer persister cells after long term treatment with TMZ.[14] The metabolic, cell survival, and epigenetic changes that occur in cancer persisters are in response to slow cell growth characterized by the environment.[15] Along with cancer persisters, cells have proteins that are transporters that pump the attempted treatments out of the tumor.

ATP binding cassette (ABC) transporters are efflux transporters that pump out different chemotherapeutics. Analysis of their expression can help guide the understanding of multiple drug resistance (MDR) in multiple cancers and patients . ABCB 1, a specific ABC transporter protein, has many different drugs that act as a substrate but the major one is TMZ.[16] Expression of this protein indicates that the GB will be less responsive to the standard of care and have a high potential to reoccur. These ABC transporters and their expression may be increased in recurrent GB and that is why they are less likely to have a response to treatment.



Schematic 1.2: *Major Players of Immunosuppression in Glioblastoma* (Made through Biorender)

The tumor microenvironment in GB and recurrent GB is highly immunosuppressive and suppresses anti-tumor activity led by immune effector cells. There is an increase in regulatory T lymphocytes (Tregs) in the tumor microenvironment (TME) and peripheral blood. Immunosuppression is mediated by heightened amounts of Tregs expressing FoxP3 in the peripheral blood which inhibits immune responses and antitumor activity.[17-23] The increase in circulating Tregs reduces the amount of cytotoxic CD 4+ and CD8+ T cells that are viable to attack the tumor.

Experimental Therapeutics

The CDC stated statistics that illustrate that Cancer is still the second highest cause of death in the United States. Treatment is still ineffective and new methods are being created, brought into clinical trials, and advancing to the clinical setting. The push towards advancing therapy lies in methods of CAR-T therapy, and specifically targeted antibodies. CAR-T therapy has had proven success with blood cancers but has not been looked at extensively at other cancers. It would not work in the brain due to unknown risk with interactions of neurons and how it could modulate cognitive and motor function. The other form of advancement comes in target-specific antibodies. These antibodies look at targeting a specific protein or activating an immune response to attack the tumor.

Table 1:1- Approved Antibodies for Treatment of Cancer (Cancer Research Institute)

Drug	Trademark Name	Target	Disease
Alemtuzumab	Campath	CD 52	Leukemia
Bevacizumab	Avastin	VEGF	Brain, cervical, colorectal, kidney, liver, lung, and ovarian cancer
Cetuximab	Erbitux	EGFR	Colorectal cancer and head & neck cancer
Daratumumab	Darzalex	CD 38	Multiple myeloma, colorectal, esophageal, liver, lung, & stomach cancer
Rituximab	Rituxan	CD 20	Leukemia and Lymphoma
Tafasitamab	Monjuvi	CD 19	Lymphoma
Trastuzumab	Herceptin	HER 2	Breast, espohageal, and stomach cancer

Table 1:1 illustrates the current approved monoclonal antibodies that target specific pathways that are promoted in cancer to

increase cell proliferation and cell migration. As shown by the chart the only one approved for glioblastoma, the brain cancer, is Avastin. This chart demonstrates that there is a need to develop more therapeutic options for glioblastoma. Further looking into other pathways and mechanism of targets to block could improve the efficacy of treatment and provide patients with more options tailored to their medical needs.

Galectin-1's Role in Cancer and Immunosuppression

Galectin-1 (Gal-1) is a lectin which is a carbohydrate binding protein that has a high affinity for binding β- galactosides. The composition of the protein includes two subunits of a total size of14.5 kDa (135 aa) that reside in a dynamic dimerization equilibrium.[24] Gal-1's structure is influenced by 2 anti-parallel β-sheets with a conserved topology of a carbohydrate recognition domain.[25] Gal-1's CRD contains a high affinity to bind LacNAc-bearing structures via van der Waals interactions and hydrogen bond formation.[25] The binding is mediated by key amino acids that include His45, Asn47, Arg49, Val60, Asn62, Trp69, Glu72, and Arg74.[25] Gal-1 is considerably upregulated and overexpressed in inflammatory macrophages, immunosuppressive DCs[26-28], activated T and B cells[29-30], CD4+CD 25+ Tregs and uterine NK cells[31-32]. Gal-1 regulates the immune effector cell populations previously stated by interactions with properly glycosylated cell receptors that

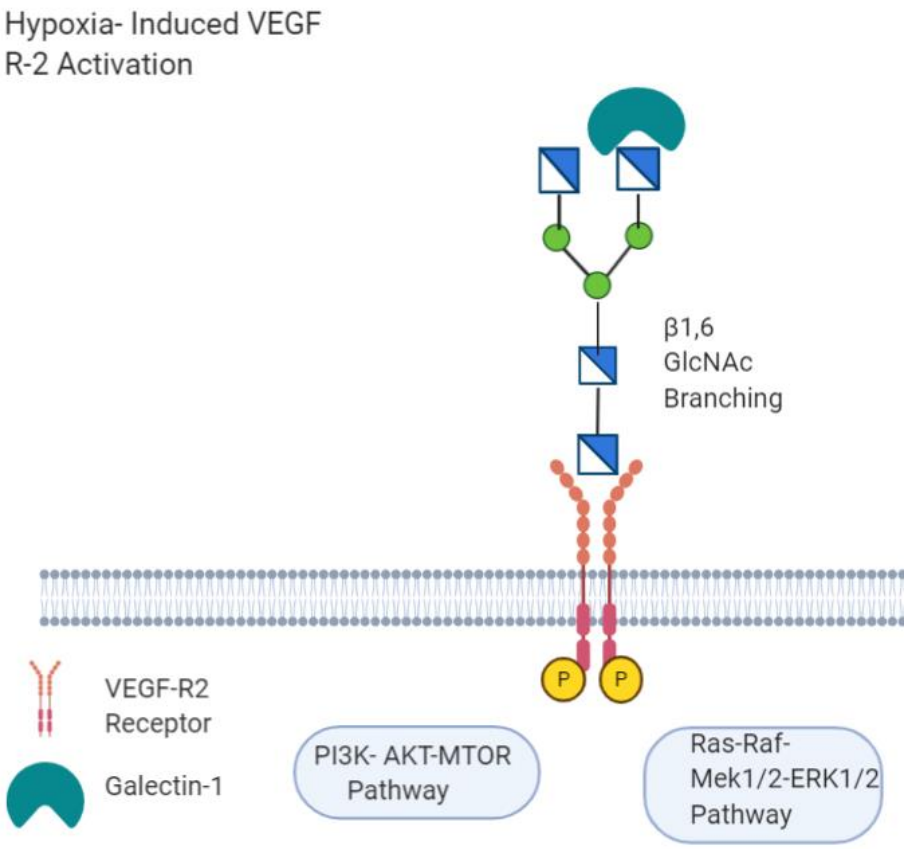
include CD 45, CD 43, CD 3, CD 2, CD 4, CD 7, CD 69, and pre-B cell receptor (pre-BCR).[33-40] Through cellular interactions stated in paragraphs below, Gal-1 has emerged as a novel regulatory checkpoint that positively influences immune evasive programs of cancer cells' by inducing T-cell exhaustion, limiting survival of T-cells, favor an expansion of regulatory T-cells, de-activate natural killer (NK) cells, and polarize myeloid cells towards an immunosuppressive phenotype.[24] Gal-1 has many implications in adaptive and innate immunity. Attached to the cell membrane Gal-1 selectively promotes apoptosis of Th 1 and Th 17 cells[41], induces Il-10 secretion[42-45], inhibits T-cell trafficking[46]and decreases nitric oxide (NO) production by macrophages.[47-48]

Gal-1 alters T-cells viability by creating disruptions that promote apoptotic factors and limit efficiency of TCR signal transduction. The Gal-1 receptors CD 3, CD 4, CD 2, CD 45, GM1, and CD 43 impact TCR signal transduction through reorganization at opposite poles during synaptogenesis and distal pole complex formation.[37-40,49-51] The lectin's immunoregulatory capabilities may result from its capacity to modulate TCR signal transduction, T-cell synaptic organization, and T-cell polarity. Gal-1 also acts as a TCR antagonist and limits sustained TCR signaling during continued CD 8+ T-cell activation.[52] Gal-1 acts as an autocrine negative regulatory of CD 8+ burst size and provides concrete identification

of modulation of the TCR ligand binding in time of T-cell activation.[52] Gal-1 regulates CD 8+ and CD 4+ T cell populations while increasing the percentage of regulatory T-cells (Tregs). The protein engages with the receptor CD 45 and instructs the T-cell to a regulatory T-cell signature which is characterized by high Il 10 and Il 21 expression.[53] Gal-1 modifies the c-Maf/aryl hydrocarbon receptor pathway in these instances. [53] Gal-1 alters T-cell fate and promotes apoptotic factors for eventual T-cell death. Gal-1 mediated cell death transpires independent of caspase activation by nuclear translocation of mitochondrial endonuclease G which is not accompanied by cytochrome C- release.[54] Gal-1 mediates t-cell viability by selectively deleting through apoptotic or TCR disruption in CD8+ and CD 4+ cells while simultaneously increasing the populations of TREGS. Gal-1 induces apoptosis in CD 8+ and CD 4+ t cells through disruption of TCR signal transduction, while increasing TREGS which induce expression of Il-10 and Il-21 expression activating a M2 phenotype in macrophages and promoting an anti-inflammatory response in the immune effector cell populations. These mechanisms lead to a heightened immunosuppressive environment for the cancer to thrive in and grow undetected.

Cancers promote a tumor microenvironment that is highly hypoxic and lacks adequate oxygen supply. Recent findings

suggest hypoxia increases the amounts of b1-6GlcNAc-branched N-glycans and poly-LacNAc structures, reduces a2-6 sialylation, and induces slight changes in asialo-core-1 O-glycans in comparison to normal healthy oxygen conditions[55] Refractory tumors occur even in the presence of treatment with Anti-VEGF mAb and in these instances Gal-1 expression increased. The recent findings showed that Gal-1 maintained activation of VEGF activated angiogenesis and proliferation pathways through over-expression phospho Akt (Thr308), Akt (Ser473), and Erk1/2.[55] Overexpression of Gal-1 reduces sensitivity to Anti-Vegf treatment by keeping the PI3K-AKT-MTOR and Ras-Raf-MEK-ERK pathways open. Gal-1 keeps these pathways open by interactions with VEGFR2 through N-glycosylation-dependent mechanisms.[55] These studies show that not only is Gal-1 promoting an anti-inflammatory response in the tumor microenvironment, but Gal-1 is also keeping angiogenesis and proliferation pathways active through complex N-glycan interactions with cell surface receptors like VEGF-A. Schematic 1.3. is below to help illustrate the binding of VEGF by Gal-1 through complex β 1,6 N glycan branching.



Schematic 1.3. *Galectin-1 Complex N Glycan Branching to VEGF* (Made through Biorender)

Role of Hypoxia in the Tumor Microenvironment

Hypoxia plays a critical role in helping to establish tumor angiogenesis and helping to create a tumor microenvironment that favors the metabolic switch of cancer cells from oxidative phosphorylation to anerobic glycolysis. Hypoxia inducible factor 1 (HIF-1) is made up of two subunits, HIF-1α and HIF-1β. HIF-1α is oxygen dependent and in normal conditions hif-1 α is degraded by the proteosome by being marked with a hydroxyl group (OH) for

degradation by proline-hydroxylase-2 (PHD-2) and by vonHippel-Lindau (VHL)-ubiquitin ligase complexes.[56-57] As tumor cells proliferate limiting the quantity of oxygen in the microenvironment, hif-1 α stabilizes and is no longer marked and degraded. Hif-1 α stabilization leads to binding of Hif-1α to Hif-1β generating the Hif-1 complex. The Hif-1 complex binds to the binding site which consist of the core sequence 5'-CGTG-3' of the hypoxic response element (HRE) domain that sits on regulatory regions of target genes to activate expression. Over 70 known genes that play roles that include angiogenesis, cell proliferation, apoptosis, and cell stemness are transcriptionally regulated by Hif-1 complex.[58] Activation of the complex leads to an increase in VEGF expression supporting angiogenesis and the reduction of oxygen in the environment making treatments such as chemotherapy and radiation less successful at attacking GB.

Role of Radiation in Cancer

Radiation is a treatment technique that has been used since the advancement of science and computer technology. Through molecular biology techniques radiation has been shown to cause single and double strand breaks in DNA which can leads to signals of apoptosis and cell senescence. Radiation decreases oxygen in the microenvironment leading to an increase in HIF-1 activity and

high doses can increase the glycan environment surrounding the tumor.

Chapter 3

Introduction

Gaining knowledge from studying human tumor samples, specifically GB, is essential to further understanding of the environment and protein expression through the tumor. Our lab has access to human samples that we can analyze, and it gives us a capacity to analyze if certain treatment methods are worth pursuing. Previous research indicates Gal-1 plays a role in helping establish an immunosuppressive tumor environment and leads to progression of cancer. The purpose of these experiments, Gal-1 expression profiling and tumor staining, was to gain a better grasp on the tumor microenvironment and characterize Galectin-1 expression in patient samples. High Gal-1 would validate the notions that this protein plays some sort of role in GB and that it is worth being investigated as a potential therapeutic target.

Materials and Methods

Human samples were supplied through a Neurosurgeon that is in our department (Department of Neurosurgery). The samples were placed on ice and were immediately placed in -80°C freezer until

further use. All collection methods followed approved IRB protocols.

Western Blot

Tissue was cut into smaller pieces with scalpel and suspended in 500 µl of Homogenizing Buffer (2 mM EDTA, 50 mM Tris-Cl (pH 7.5), 0.5 mM DTT)(dithiothreitol)). Tissue was sonicated and 5µl was pipetted and placed in a glass vial filled with 1ml of DI water. After that step, the glass vial was filled with 1ml of Coomassie assay buffer and vortexed. 200µl was placed in a 96-well plate reader along with standards of 0, 2, 5, 10, 20, and 40 µg of BSA (concentration 1µg/µl) and the plate was read at 495 nm. Protein content was calculated, and tissue was diluted to 1µg/1ml with 500 µl of Sample Buffer and Homogenizing Buffer + Sample Buffer + 1 % HB+SB Bromophenol. The tubes were stored in -80°C freezer until was ready to use.

Tubes were taken out of freezer and placed in warm water. The tubes were then vortexed. Bio-rad Pre-cast TGX 4-15% Tris/Glycine Page Buffer 15 well (15µl) were placed in a electrophoresis chamber filled with SDS running buffer. Each well was filled with 7.5 µl of specific human sample protein, and the system ran at 125 volts for 1.5 hours. Once the protein ran to the end of the gel, it was placed on a PVDF membrane sandwiched between to filter papers between sponges that were soaked in Transfer buffer. The PVDF membrane in the Transfer buffer was

ran at 12 volts for two hours. After this the membrane was removed and placed in a blocking solution of 2% non-fat milk in TBST. The membrane was blocked for 1 hour, then the blocking buffer was discarded and replaced with primary antibody at a concentration of (1:2,000) in 2%non-fat milk TBST. The membrane was incubated overnight at 4°C. After incubation, the primary antibody solution was discarded and saved for further Western Blots. The membrane was washed three times with TBST for 12 minutes each. TBST was discarded and the membrane was placed in the secondary antibody (1:5000) 2 % non- fat milk TBST solution. The secondary antibody used is dependent upon the primary antibody. The secondary antibody was discarded after an hour of incubation at room temperature. The membrane was washed in TBST and then taken to a Chemi Doc imager. ECL was prepared by adding equal solutions of solution A and solution B to a 15 ml tube wrapped in tinfoil to cover it from light. 1 ml of ECL was placed on each membrane once placed an imager. Image was saved to flash-drive for data- analysis.

Cryostat Sectioning

Tumor samples were sliced into small pieces using a scalpel and one small piece was taken and placed in tissue freezing medium (TFM) and allowed to freeze in the medium in the -80° freezer. Once ready the frozen TFM and tissue were mounted on a cryostat

specimen chuck. The tissue was sliced at an approximate width of 12 µm and tissues were placed on Fisher Brand Super frost Plus pre cleaned slides. Slides were correctly labeled and stored in -80°C freezer until ready to go through immunofluorescence staining.

Immunofluorescence Staining

Slides are taken out of the -80° C freeze and allowed to come to room temperature by incubating for 45 minutes to an hour. Then slides are immersed in 95% EtOH for 10 min followed by two washes in PBS (1X) for 2.5 minutes each. The slides are moved to a solution of 4% paraformaldehyde dissolved in PBS (1X) where they are immersed for 15 minutes followed by three washes with PBS (1X) for 2.5 minutes each. Following the washes with PBS (1X) the slides are immersed in 0.2% Triton X-100 dissolved in PBS (1X) for 5 minutes followed by another three washes of PBS (1X) for 2.5 minutes each. 100µl/slide of 4% serum in PBS(1X) is applied to each slide for an hour for blocking. The serum is dependent upon the species of the secondary antibody. In our experiments the secondary antibody is goat, so the serum is 4% goat serum in PBS (1X). The 4% goat serum in PBS (1x) is wiped away from the edges not touching the tissue by a Kimwipe, then 100µl of the primary antibody(1:500 dilution) is applied to each slide for 2 hours at RT followed by three washes of PBS (1X) at 2.5

minutes each. After the three washes with PBS (1X), 100µl per slide of secondary antibody (1: 500 dilution) in 4% goat serum in PBS (1X) is applied to the slides for 1 hour at RT. Application of the secondary antibody and the following steps must occur in the dark. Ensuing secondary antibody incubation, the slides are washed three times with PBS (1X) at 2.5 minutes each. Kim wipes are used to dry the edges around the tissue in the slides and a plastic Pasteur pipette is used to incorporate 1-2 droplets of Vectashield+ Dapi onto the slides. Cover slips follow this process and are placed over the mounted stained tissue. Slides can be kept for 2-3 days and are imaged using an Olympus.

Statistical Analysis

For IHC staining 12 images were taken with each of the constraints that were ROS 1 positive and ROS 1 negative. Western Blots conducted included 10 samples for each of the patients and 10 samples for the normal brain tissue which was taken from healthy individuals with no current state of mental disease or disorder. Data illustrated in this thesis for human patient samples illustrates the expression conserved through multiple exposure of analysis from the samples where n is greater than or equal to 10.

Results

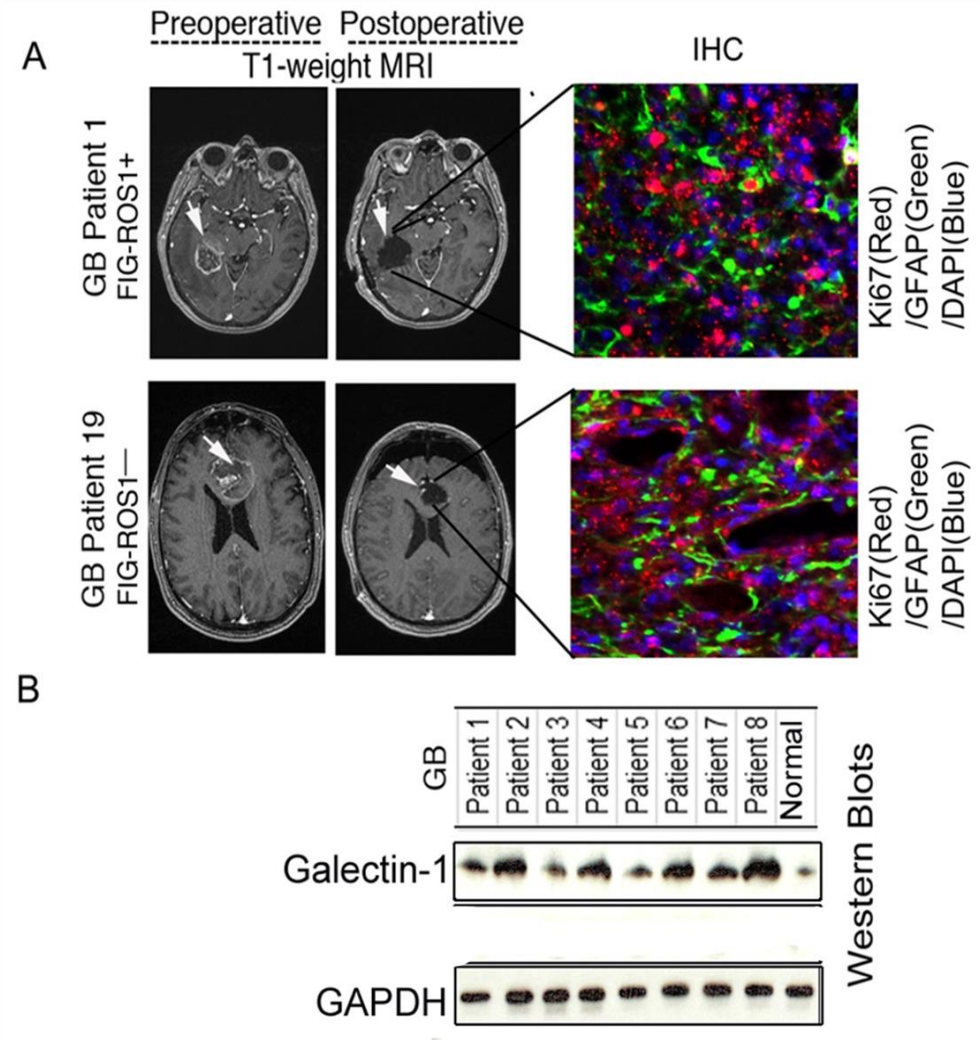


Image 3.1: *Staining and Expression Analysis of GB Patient*

The sample size for the staining and western blots was 20 patients and the normal tissue analysis was 12 patients. There were 12 images taken from each patient for the IHC of Ki-67, GFAP, and Dapi.

Discussion

The results indicate that Gal-1 is overexpressed in GB patients. This overexpression leads us to conclude that Gal-1 must play a role in establishing the tumor and the tumor's microenvironment to prevent effective treatment. Immunofluorescence staining of the tissue indicates that the GB tumors have an extremely high rate of proliferation (Ki-67), and expression of Glial fibrillary acidic protein (GFAP). GFAP is a commonly used marker to get an understanding of the severity of the tumor. The analysis of human samples drives our lab to look further into Gal-1 expression by conducting various in vitro and in vivo assays. Our next steps will be to understand Gal-1 expression in endothelial cells and test its feasibility as a therapeutic through in vitro drug assay methods.

Chapter 4

Introduction

From analysis of Glioblastoma patients there was an increase in Gal-1 expression compared to healthy tissues. These results indicated that this protein has some sort of role in supporting tumor growth. The goal of these experiments was to validate Gal-1 inhibition as a potential treatment in an in-vitro model while comparing it to VEGF inhibition and in conjunction with VEGF inhibition. The second goal of the experiments was to design a co culture system with GB cells and HUVEC cells. Previous literature suggest that Gal-1 has strong roles in the extracellular matrix

(ECM) and endothelial cells. Gal-1 may act similarly to VEGF in increasing endothelial cell proliferation leading to increased angiogenesis that supports tumor function and growth. These experiments aim to answer these questions and provide more background into relationships Gal-1 has with different factors of Glioblastoma.

Materials & Methods

In-vitro Drug Treatment of GB Cells

The Glioblastoma cell lines, 43 RG 32 28 24 which were given to us by the Cleveland Clinic, were either not treated or treated with Bevacizumab, OTX 008, or Bevacizumab + OTX 008. Cells were grown until approximately 70% confluency in the flask until treatment started. The combinational treatment of Bevacizumab + OTX 008 started with treatment of Bevacizumab on the first day followed by OTX 008 on the 2nd day. On the 5th day cells were scraped from the flask and inserted into a 15ml conical tube with 10 ml of media. The cells were spun at 5,000 rpm for 5 minutes, and the media was discarded, and the cells were frozen in -80°C freezer until a Bradford assay was ready to be conducted for protein estimation.

MTT Assay

A MTT solution was created at a concentration of 5mg/ml dissolved in PBS. Preparation of MTT solvent was created with 4 mM HCl, 0.1% NP40 in isopropanol. Cells were scrapped from 75cm^3 and spun at 4,000 g for 5 min. Then supernatant was poured off and the cells were suspended in 100 µl of serum free media. After suspension 50 µl of cells + serum free media were pipetted into the 96 plate well. 50 µl of MTT was placed in each well. The 96 well plate was incubated for 3 hours at 37 ºC. Add 150 µL of MTT solvent into each well. Wrap plate in foil and shake on an orbital shaker for 15 minutes. Read absorbance at OD=590 nm.

Western Blots

Cells were lysed and protease inhibitors were utilized. Cells were suspended in 100 µl of Homogenizing Buffer (2 mM EDTA, 50 mM Tris-Cl (pH 7.5), 0.5 mM DTT)(dithiothreitol)) After suspension cells were transferred to tubes with correct labeling. The suspended cells were sonicated. Glass tubes were filled with 1 ml of deionized (DI) water, 5 µL of suspended protein+ HB, 1ml of Coomassie assay buffer, and then vortexed. 200 µl of solution from the glass tube was taken and plated on a 96 well plate. The standards for the Bradford Assay were 0,2,5,10,20,40 (µg/µl) BSA duplicated and plated. Two solutions of 200µl were taken from each glass tube and then averaged and calculated to make 1µg/ 200µl solutions with HB+SB (Bromo-phenol) added for purpose of western blots. Gels

were loaded and run at an hour and half at 100 volts. The gels were then sandwiched between a PVDF membrane and two membrane filters and placed in transfer buffer and run at 12 volts for two hours. Then membranes were placed in blocking buffer (2% non-fat milk in TBST) for one hour. Then the selected primary antibody was diluted 1:2,000 in 2% non- fat milk TBST. The blocking buffer was discarded and the membranes were placed in the primary antibody cocktail overnight at 0 to -4°C on a shaker. The next day the membranes are allowed to come near room temperature then are discarded and washed three times for 12 minutes with TBST. After that the membranes are placed in a secondary antibody cocktail that includes the selected secondary antibody at a concentration ranging from 1:5,000-1:8,000 in 2% nonfat milk and TBST. The membranes are incubated at room temperature for an hour. The membranes are washed another 3 times with TBST and then are ready to be imaged by a Bio-Rad ChemiDoc imager. Membranes are pulled from their containers one by one and drained of TBST and placed on the screen of the imager. ECL is prepared ahead of time with equal parts of solution A and solution B. 1.5 of ECL reagent is pipetted on the membrane, and then the membrane is imaged for protein expression of selected primary antibody.

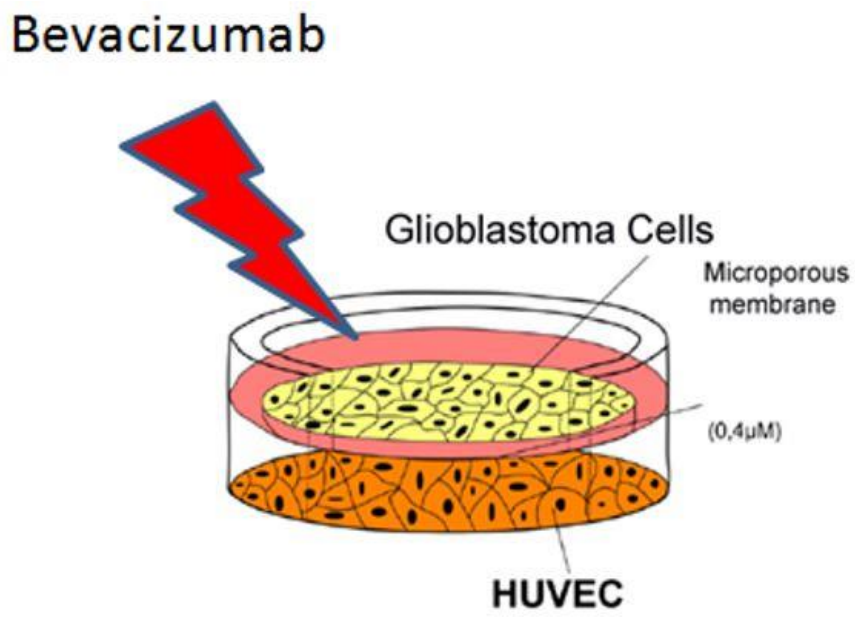
Caspase 3 Assay

Cells that were undergoing treatment were lysed and protein estimation calculations (Bradford Assay) were conducted. Approximately 250µg of protein of each sample were taken for use in the caspase 3 colorimetric assay (Sigma Aldrich). The microliter quantity that gave 250µg of protein was taken for each sample and placed in the specific well. The cell treatments calculated came from triplicate of the treatments conducted. After the cell lysate was placed in the specific wells, 980µl of 1x Assay Buffer was added to each well. The Assay Buffer was formed by diluting 10x assay buffer to the correct volume with the 17 megaohm water provided in the kit. 10µL of Caspase 3 substrate was then added to well. The caspase 3 substrate was dissolved in DMSO and diluted in 1x assay buffer to bring the Caspase 3 substrate from 20mM down to 2 mM. The plate was then covered and incubated at 37°C overnight. The next day the absorption of the plate was run at 405 nm.

Co-Culture Proliferation Assay with Bevacizumab

Co-culture assays helped to establish the role of Galectin-1 as a factor similar to VEGF in its promotion of angiogenesis. For the co-culture the bottom layer of cells on the system were Human umbilical vein endothelial cells (HUVEC) and the system was set up once the HUVEC reached approximately 20,000 cells. The number of cells was validated through cell counting on a hemocytometer.

The cells were counted 3 times at each day indicated with the experiment run in triplicate. P values did not exceed .05. The glioblastoma cell line was plated on a 0.4µm filter system position above the endothelial cells. Schematic 3.1 shows the set up of the co culture system. Once the Glioblastoma cells were plated on the porous filter membrane and were treated either with either no treatment, dose of Bevacizumab, or dose of Bevacizumab and OTX008. Following this treatment of the Glioblastoma cells, the quantity of HUVEC is counted at the intervals of initial (0 days), 3 days, 7 days, and 10 days. Counting was done through trypan blue dying for cell viability and counted with the utilization of the square system of the hemocytometer. The three constraints of treatment methods went as follows: untreated, Bevacizumab, and Bevacizumab+ OTX008. There were 4 co culture systems of each treatment method to be able to effectively count cells at 0, 3, 7, and 10 day periods. After treatment was finished. Cells were blocked with protease inhibitors and lysed to run Westerns to look at Galectin-1 expression.

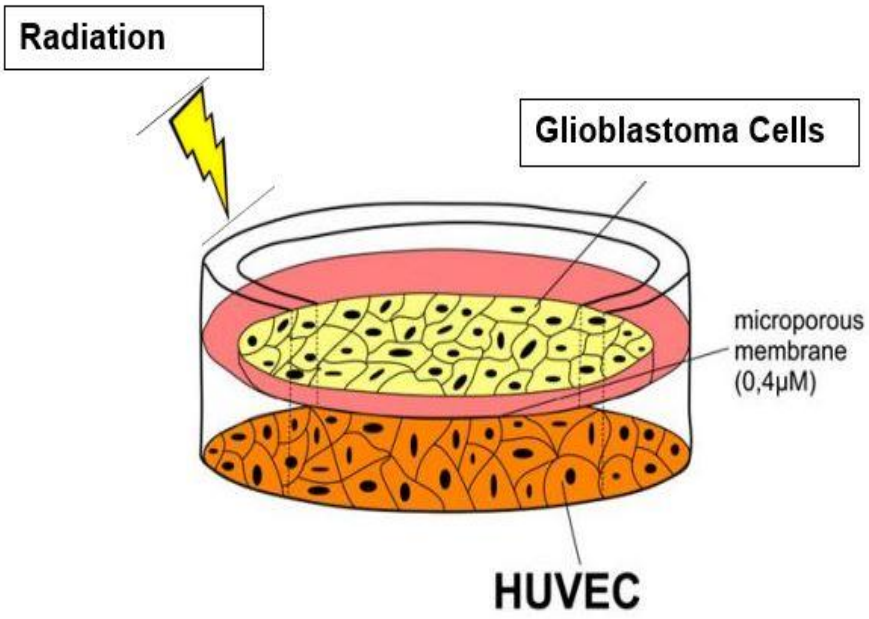


Schematic 4.1: Treatment of Bevacizumab in Glioblastoma Cells Co Cultured with Endothelial Cells

Co-Culture Proliferation Assay with Radiation

A co-culture assay was set up in a similar style to that of the previously describe co-culture with Bevacizumab treatment, but this time focusing on radiation. Scientific literature shows mixed reviews on the influence of radiation in cancer. Previous research indicates that radiation, more specifically higher doses of radiation lead to healthy cells and tissue surrounding the tumor turning into necrosis which allows for tumor growth and helps in making a larger barrier to thwart treatment options. This study was done to further develop and comprehend the relationship between radiation and Gal-1 expression. Glioblastoma cells were grown and plated on a 0.4µm porous membrane that was placed in a proximity dependent manner above the endothelial cells. The endothelial cells were

plated before the Glioblastoma cells on the bottom of the petri dish at a population of approximately 20,000 cells. 6 gy of radiation was



given consecutively (3 times total) after 72 hours of the co culture being in place. The treatment constraints were untreated, radiation, radiation and OTX008 together. Endothelial cells were stained with trypan blue to validate cell viability and counted utilizing a hemocytometer. Endothelial cells had protease inhibitors added and cells were lysed through sonication for purpose of protein estimation and Western Blot analysis.

Schematic 4.2: Treatment of Glioblastoma Cells with Radiation Co cultured with Endothelial Cells

Cell Counting with Hemocytometer

The goal of the co-culture assays is to analyze different treatment options and their influence on angiogenesis by looking at endothelial cell proliferation. Endothelial cells must be counted to effectively analyze their characteristics in the presence of treatment. The device used is called a hemocytometer and has little grid like squares that can be utilized for a full estimation of the cell count for different subsets. The cells were counted 3 times each where n=3. By having 9 counts of cells it gives a better estimation of cell count through the triplicate of experiments conducted.

Statistical Analysis

All studies conducted are at constraints where n is greater than or equal to three. For cell proliferation assays, cell counts were conducted three times for each time point and the Glioblastoma cell co culture with endothelial cells was at n is greater than or equal to three. Each of the figures indicates the values calculated between time points and multiple stages of data collection. The percentage of standard of error for cell counting did not exceed .05 for any of the constraints.

Results

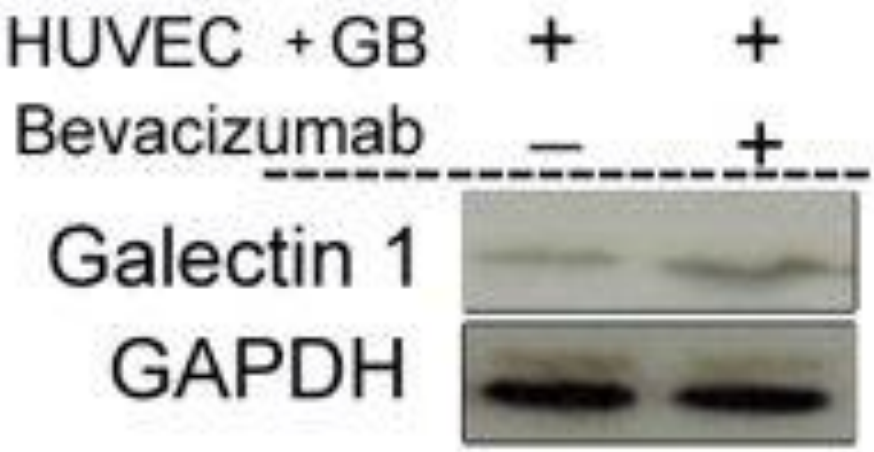


Image 4.1 Gal-1 Expression in Bevacizumab Treated Co-Culture

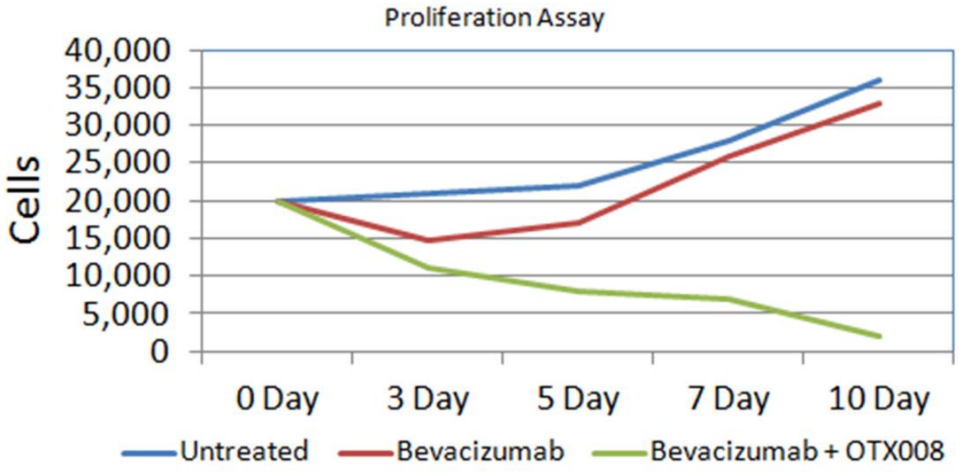


Image 4.2 *Bevacizumab and OTX 008 HUVEC Proliferation*

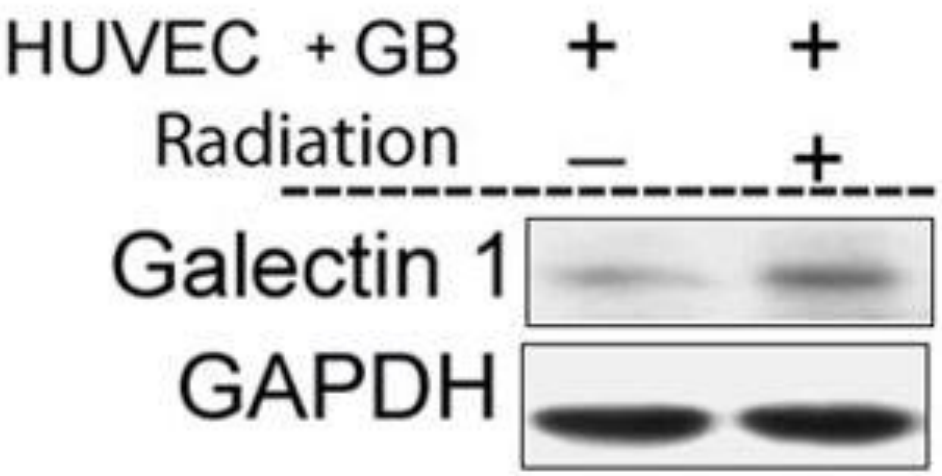


Image 4.3 *Galectin-1 Expression Radiation Treated Co-culture*

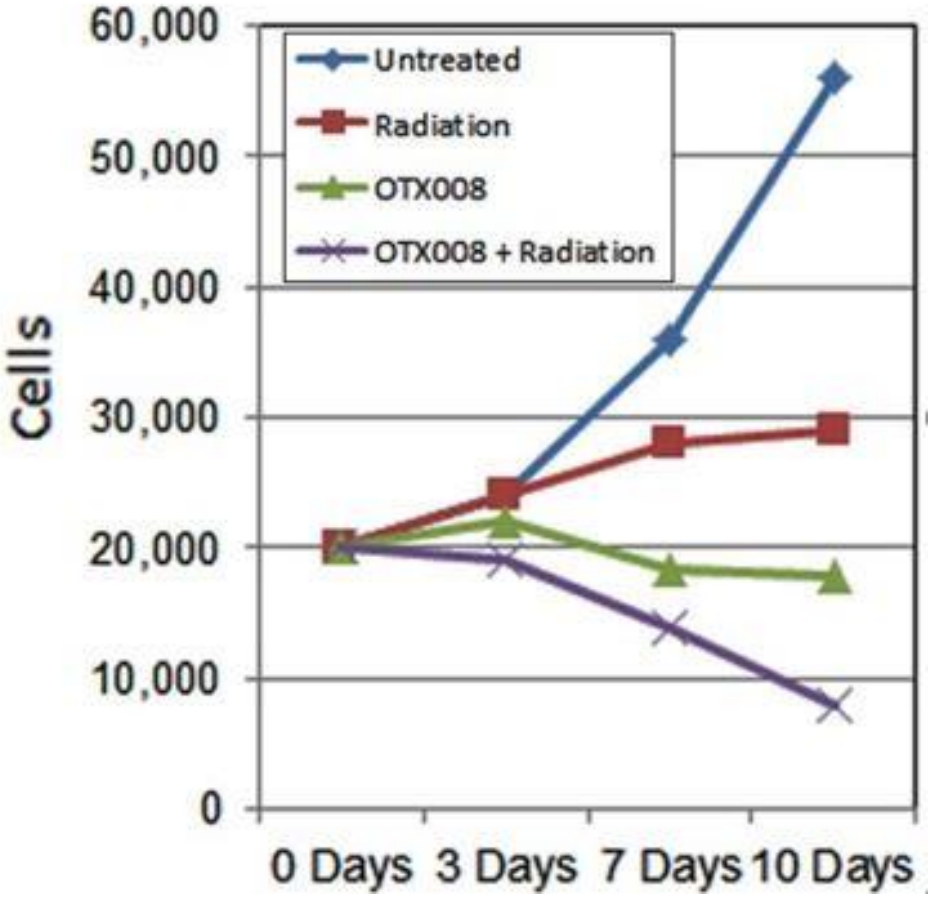


Image 4.4 *Radiation and OTX 008 HUVEC Proliferation*

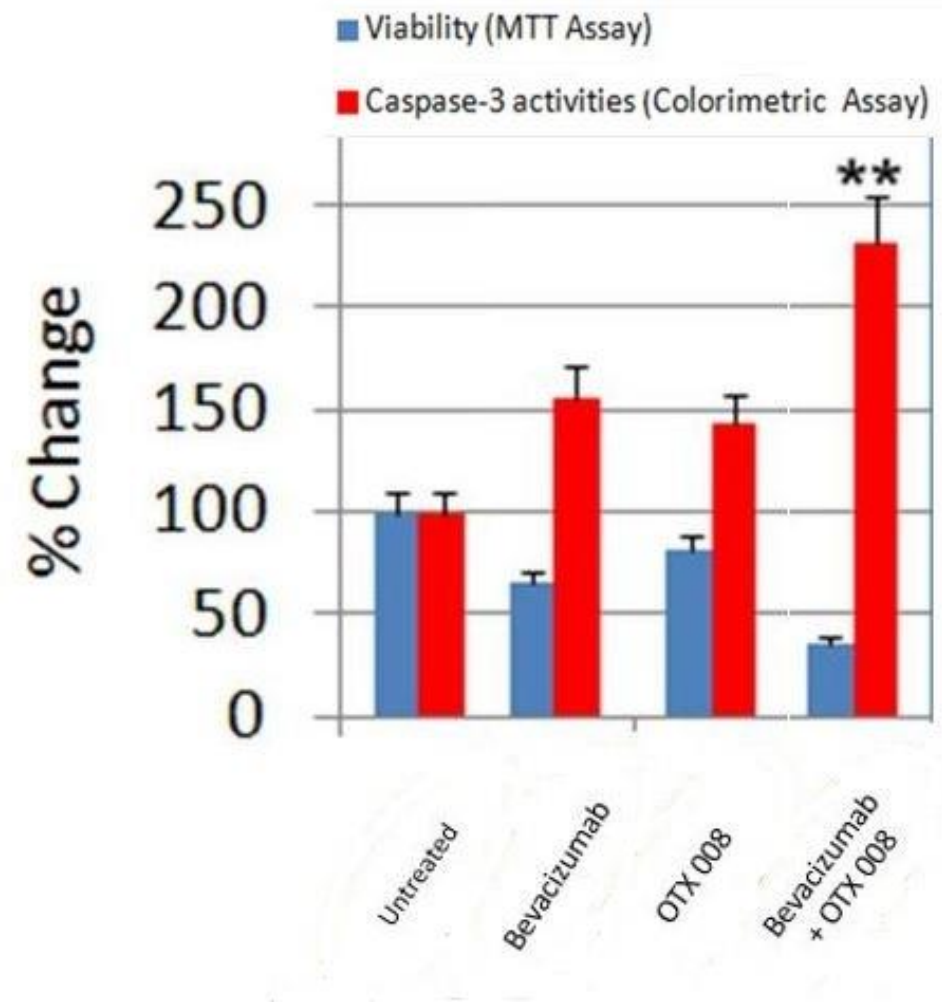


Image 4.5 *Cell Viability and Apoptotic Activity*

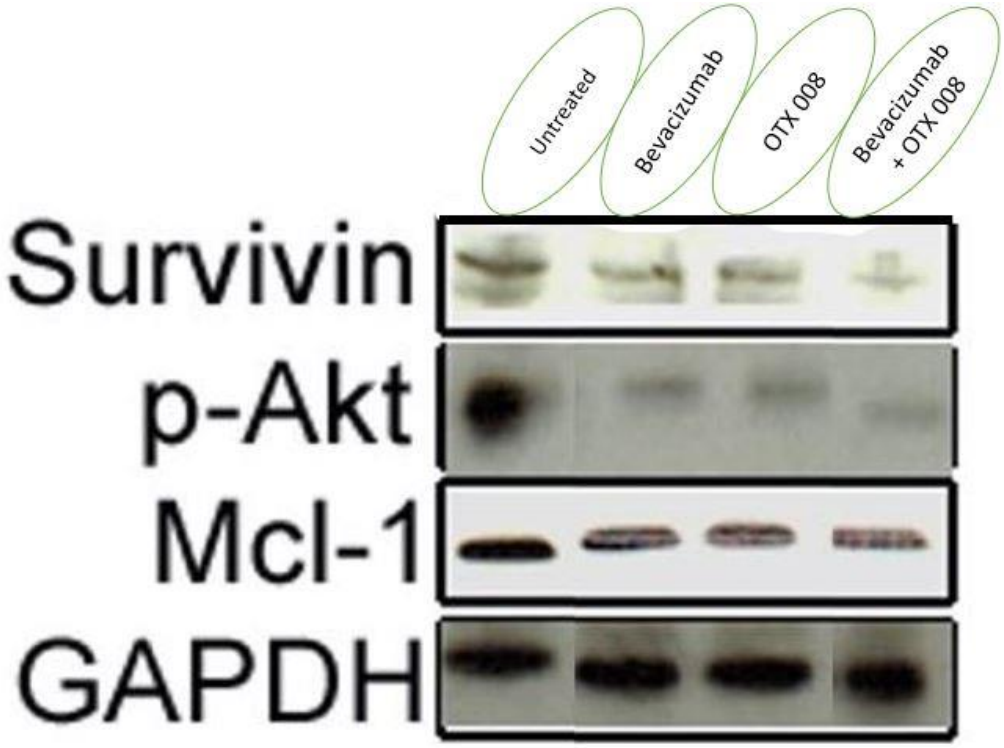


Image 4.6 *Western Blot Analysis*

Discussion

The results indicate that there is an increase in apoptotic activity (Caspase 3 assay) and decrease in cell viability (MTT) assay with regards to Vegf inhibition (Bevacizumab), gal-1 inhibition (OTX 008), and a combination of Vegf + gal 1 inhibition (Bevacizumab + OTX 008). The sharpest decrease comes in the form of the combinational treatment, indicating that it might be the most feasible way to reduce the functionality of the tumor cells. At the same time Gal-1 expression is influenced by common treatment methods that are generally given in the clinic for either GB or recurrent GB. Both co culture assays with Bevacizumab and

radiation showed a sharp increase in Gal-1 compared with the control. These increases amounted to approximately 18-24% in both cases. These results specify Gal-1 expression and the alteration of Gal-1 expression that takes place in blood cells. A direct correlation was portrayed by Gal-1 expression and endothelial cell proliferation in response to treatments. This direct correlation has the potential to be a mechanism that tumors incorporate to keep vasculature and promote angiogenesis even in the presence of treatment. From this data, we decided to go a step further and investigate the response to a mouse glioblastoma cell line injected into a C57BL6/J mouse.

Chapter 5

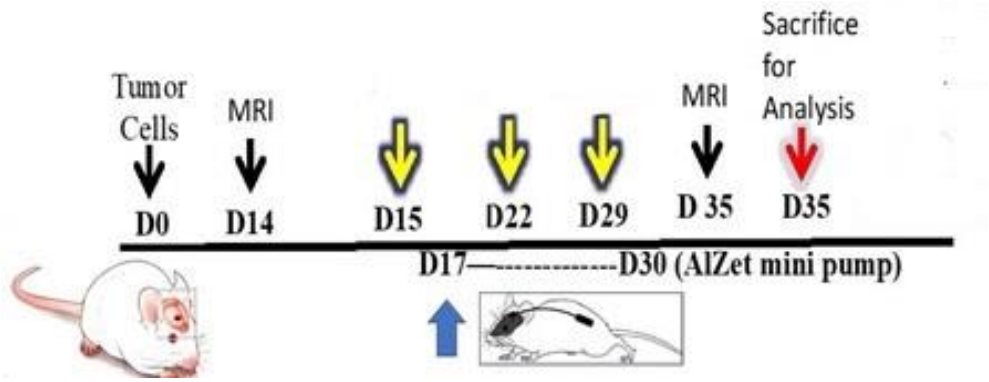
Introduction

The goal of this study was to look deeper at the potential of Gal-1 inhibition with or without conjunction of VEGF inhibition. The in-vitro studies drug studies were for validation of the concept of Gal-1 as a potential therapeutic target. The next step was to look at the potential therapeutic or combinations of the potential therapeutic for the treatment of GB in a relevant mouse model. Mouse models provide further validation of therapeutic success due to their more realistic stimulation. For the mouse, you have a similar microenvironment that holds microglia, astrocytes, and similar neuronal structure to the human brain. This method of

experimentation gives a better indication of the effectiveness of the therapeutic.

Materials and Methods

Schematic 5.1: Experiment Design of Mouse Model



Gl 261 (mouse glioblastoma cell line) were grown to 30% confluency. Cells were washed three times with PBS and then centrifuged at 5,000rpm for 5 minutes. Following that cells were suspended in 5 µl of PBS, and ready for use of injection.

Bevacizumab Recurrent Model

The Bevacizumab recurrent model had a slightly different timepoint then the previous described schematic and detail of the mouse Glioblastoma model. The Alzet mini pump was placed in the same time period shortly after implantation of the Gl 261 tumor cells. Anti-VEGF therapy started immediately on the first day. After 14 days Magnetic Residence Imaging (MRI) was conducted on the mice for verification of tumor. Only the mice with validation of tumor growth

were selected for treatment with OTX 008 and the bed of the Alzet mini pump was replaced with OTX 008. The goal of these studies was to see the feasibility of Gal-1 inhibition in a relevant Bevacizumab recurrent model.

Dissection of Brain

Mice were sacrificed following survival analysis. After animal sacrifice surgical scissors were used to cut through the skin covering the skull, and then used to cut the skull posterior to anterior to give a clear image of the brain. The brain was then gently pulled out of the mice to give a clear path to cut the connection of the spinal cord to the brain. From this point a scalpel was used to cut the connection, and brains were labeled appropriately in tinfoil boats. The tinfoil boats were filled with tissue freezing medium to protect the genetic profile of the brain and stored at -80°C for later use.

Cryostat Sectioning

The brains defrosted for 5 minutes before specimens were mounted on a specimen chuck. The brains were mounted in a manner shown by schematic 5.2 below. The schematic shows that mounted occurred posterior to anterior with cerebellum facing up and being the first part to be sliced. Contact with the tumor began shortly after finishing slicing cerebellum. Slices were collected on

permafrost glass slides and stored at -80°C freezer until staining protocols started.

Antibodies, Microscope, and Software

Table 5.1 *Antibodies used for Staining.*

Primary Antibody	Secondary Antibody
Santa Cruz CD 133 Rabbit pAb	Abcam Goat pAb to Rabbit Alexflour 488
Sigma Hif-1α rabbit mAb	Abcam Goat pAb to Rabbit Alexflour 489
Abcam Ki-67 rabbit mAb	Abcam Goat pAb to Rabbit Alexflour 490
Abcam Galectin 1 rabbit mAb	Abcam Goat pAb to Rabbit Alexflour 491
Sigma VEGF-A rabbit pAb	Thermo Scientific Goat Anti Rabbit Dylight 594
Mtl International Survivin rabbit pAb	Abcam Goat pAb to Rabbit Alexflour 493

Table 5.1 is meant to identify the types of primary and secondary antibodies used for the purpose of IF imaging. The device used for the imaging is the Olympus DP 80 multicolor image camera attached to a confocal microscope. The set up is inverted with the tissue slides placed face down for imaging. The software used to image and quantified the pixel area for the fluorescence excitation is cellSens by Olympus. Three images were taken on each brain with 10 tumor slices per brain. The pixel area of the fluorescence excitation was calculated as along with the standard deviation.

Immunofluorescence Staining

Slides are taken out of the -80° C freeze and allowed to come to room temperature by incubating for 45 minutes to an hour. Then slides are immersed in 95% EtOH for 10 min followed by two washes in PBS (1X) for 2.5 minutes each. The slides are moved to

a solution of 4% paraformaldehyde dissolved in PBS (1X) where they are immersed for 15 minutes followed by three washes with PBS (1X) for 2.5 minutes each. Following the washes with PBS (1X) the slides are immersed in 0.2% Triton X-100 dissolved in PBS (1X) for 5 minutes followed by another three washes of PBS (1X) for 2.5 minutes each. 100µl/slide of 4% serum in PBS(1X) is applied to each slide for an hour for blocking. The serum is dependent upon the species of the secondary antibody. In our experiments the secondary antibody is goat, so the serum is 4% goat serum in PBS (1X). The 4% goat serum in PBS (1x) is wiped away from the edges not touching the tissue by a Kimwipe, then 100µl of the primary antibody(1:500 dilution) is applied to each slide for 2 hours at RT followed by three washes of PBS (1X) at 2.5 minutes each. After the three washes with PBS (1X), 100µl per slide of secondary antibody (1: 500 dilution) in 4% goat serum in PBS (1X) is applied to the slides for 1 hour at RT. Application of the secondary antibody and the following steps must occur in the dark. Ensuing secondary antibody incubation, the slides are washed three times with PBS (1X) at 2.5 minutes each. Kim wipes are used to dry the edges around the tissue in the slides and a plastic Pasteur pipette is used to incorporate 1-2 droplets of Vectashield+ Dapi onto the slides. Cover slips follow this process and are placed over the mounted stained tissue. Slides can be kept for 2-3 days for

optimal imaging at 4°C or -80°C for longer storage and are imaged using an Olympus microscope.

Results

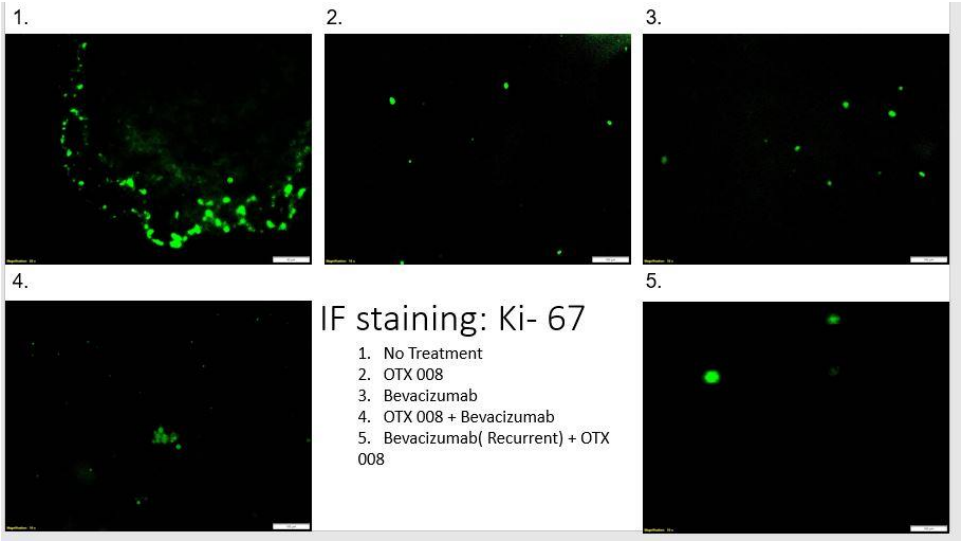


Image 5.1 *Ki-67 Cell Proliferation Analysis: Mouse Samples*

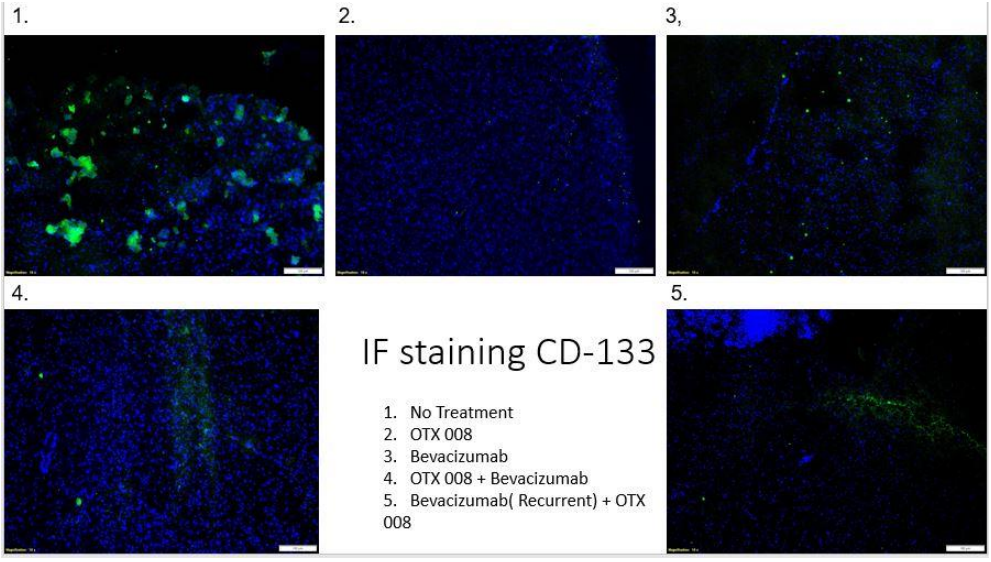


Image 5.2 *CD 133 Cancer Stem Cell Analysis: Mouse Samples*

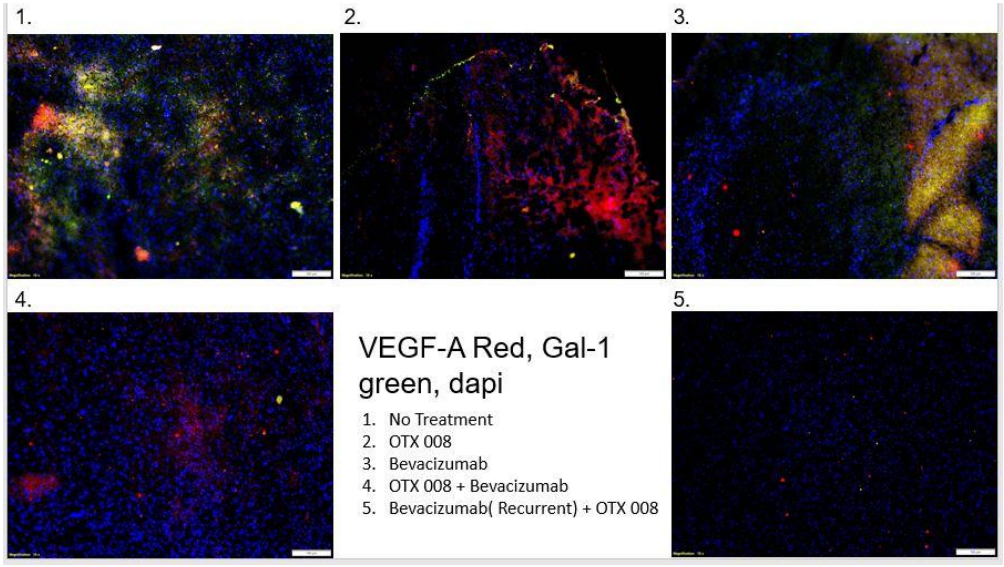


Image 5.3 *VEGF-A and Gal-1 Expression Analysis: Mouse Samples*

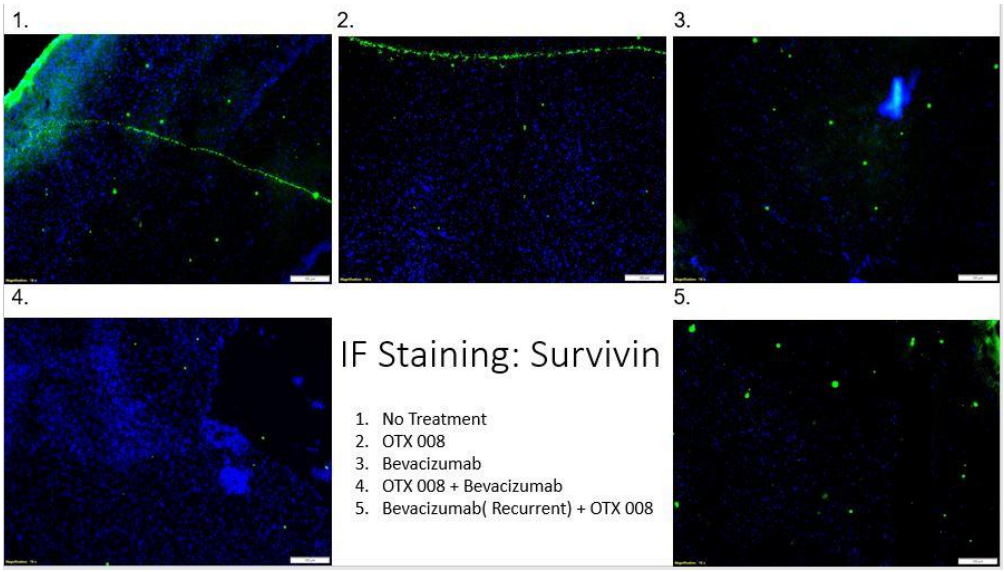


Image 5.4 *Survivin Apoptotic Activity Analysis: Mouse Samples*

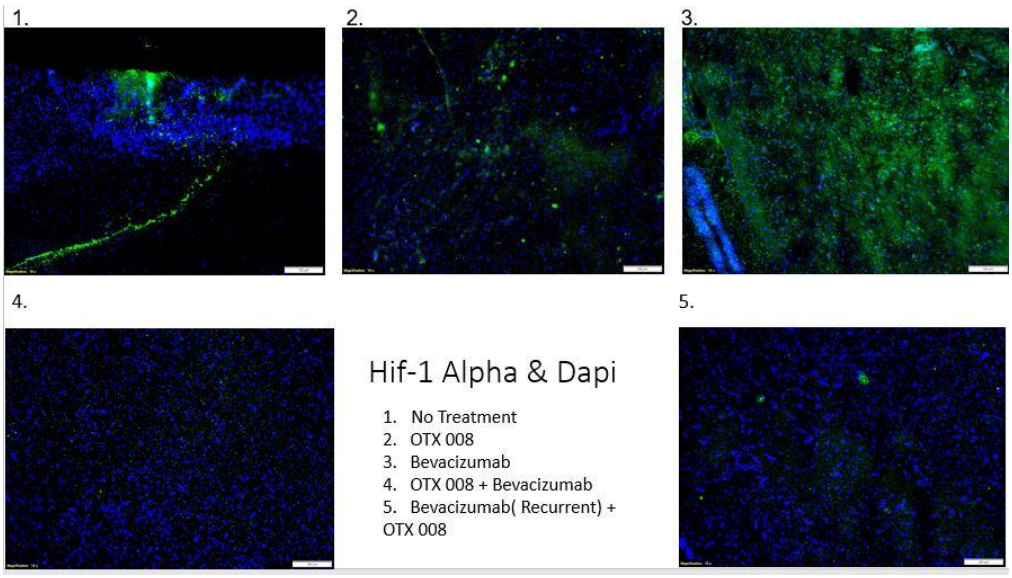


Image 5.5 *Hif-1α Hypoxia Analysis: Mouse Samples*

Table 5.2: *Ki-67, Hif-1 α, Survivin, & CD 133 Immunofluorescence Pixel Area(μm²)*

Ki-67	**Pixel Area μm squared**	**Standard Error (%)**
No treatment	1410.25	0.0017
OTX008	659.2	0.0027
Bevacizumab	904.28	0.0025
OTX 008+ Bevacizumab	402.63	0.0116
Recurrent Bevacizumab + OTX 008	1137.56	0.0066
Survivin		
No treatment	1347.42	0.0050
OTX008	910.16	0.0060
Bevacizumab	1012.23	0.0086
OTX 008+ Bevacizumab	342.43	0.0073
Recurrent Bevacizumab + OTX 008	1218.67	0.0061
CD 133		
No treatment	2063.32	0.0055
OTX008	254.81	0.0327
Bevacizumab	609.27	0.0201
OTX 008+ Bevacizumab	78.86	0.0153
Recurrent Bevacizumab + OTX 008	128.82	0.0059
Hif-1 α		
No treatment	1085.1925	0.0021
OTX008	463.27	0.0089
Bevacizumab	527.86	0.0080
OTX 008+ Bevacizumab	109.87	0.0219
Recurrent Bevacizumab + OTX 008	210.78	0.0108

Table 5.3 *Gal-1, VEGF-A, & Co-localization between VEGF-A and Gal-1 Immunofluorescence Pixel Area (μm²)*

	Pixel Area μm squared	Standard Error (%)
Gal-1		
No treatment	347.81	0.0189
OTX008	128.61	0.0172
Bevacizumab	278.41	0.0161
OTX 008+ Bevacizumab	28.27	0.0258
Recurrent Bevacizumab + OTX 008	4.63	0.0518
VEGF-A		
No treatment	420.26	0.0177
OTX008	807.23	0.0128
Bevacizumab	694.2	0.0133
OTX 008+ Bevacizumab	455.53	0.0143
Recurrent Bevacizumab + OTX 008	81.68	0.0204
VEGF-A & Gal-1 Co-localization		
No treatment	1134.24	0.0130
OTX008	81.68	0.0225
Bevacizumab	2340.56	0.0072
OTX 008+ Bevacizumab	62.83	0.0194
Recurrent Bevacizumab + OTX 008	3.47	0.0317

Discussion

Staining allows for imagery in science which can be novel and help piece together relationships of various factors between different treatment methods. The goal of these stains was to visualize hypoxia, cancer proliferation, cancer stem cell populations, VEGF-A, Gal-1, and anti-apoptotic activity across the treatment groups of no treatment, Gal-1 inhibition through OTX 008, VEGF inhibition through Bevacizumab, a combination of the two, and a recurrent VEGF model. Ki-67 showed a vast proliferation in untreated and recurrent GB. There were similar expressions of Ki-67 in

Bevacizumab treated and OTX 008 treated. CD 133 and Survivin had the highest expression in untreated while both single treatments had less but near equal amounts. The combinational treatment had the lowest expression of CD 133, Survivin, Ki-67, and Hif-1 α. This expressional analysis indicates that a dual therapy is the best option to combat the angiogenesis and hypoxia characteristics of the tumor microenvironment. VEGF-A expression was the highest in untreated and Gal-1 but there was still expression in the other models. VEGF-A has expression in the other models indicating it is still in the environment. The pixel area of fluorescence intensity illustrates the same results depicted in the images.

Chapter 6: Conclusion

The evidence collected points towards Gal-1 expression playing a role in promoting GB survival and growth. GB in the presence of standard treatment methods utilizes Gal-1 to maintain angiogenesis and hypoxia of the tumor microenvironment. Inhibition of Gal-1 leads to a decrease in cell viability (MTT assay) and an increase in apoptotic activity (Caspase 3). Endothelial cell proliferation and increase in Gal-1 expression in response to GB cells being treated with Bevacizumab or OTX 008 indicates Gal-1 expression rescues endothelial cell proliferation and angiogenesis capacities. Gal-1 keeps expression of Hif-1 complex active even in the presence of

VEGF inhibition by Bevacizumab. These results show strong direct correlations between Gal-1 hypoxia, angiogenesis, and inhibition of apoptotic activity.

These results are promising showing that Gal-1 plays an important role in maintaining the GB microenvironment and allowing for GB progression. The next steps to follow these experiments will take a closer look at the role that complex N-glycans are playing in the tumor microenvironment. Is it purely Gal-1 expression that drives recovery of angiogenesis or is it dependent upon the glycome profile of the microenvironment? A knockout with a specific antibody to block complex n glycans would enable to see if its an internal activation of Gal-1 in some sort of positive feedback loop or N-glycans allowing Gal-1 to bind and keep VEGFR2 pathways open. Next I would create a humanized mouse model with CD 34+ stem cells to implant human GB tumor samples in the brain. The focus of this experiment is to conduct survival analysis and look at populations of macrophages, NK cells, T cells, and dendritic cells. A clear understanding of the immune effector cell phenotype with or without Gal-1 inhibition will illustrate Gal-1 role in the immune microenvironment. These experiments will further identify and single out mechanisms of Gal-1 vs mechanisms of glycans in the tumor microenvironment. VEGF-A still had expression across blockage from Bevacizumab so research to identify if it binds to

other receptors or still has influence in TME could help answer questions to why Bevacizumab is not effective in patients and certain tumor types.

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