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Identification of Secretion Factors that Restructure Neural Tissue in a Model of Low Grade Glioma

Jackson Ayers
University of Connecticut - Storrs, jackson.ayers@uconn.edu

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Author: Jackson Ayers
Physiology and Neurobiology

2021 Holster Scholar

Mentor: Dr. Joseph LoTurco, Department of Physiology and Neurobiology

Abstract

This project revolves around the study of the changes to the tissue adjacent to the growth of a Low Grade Glioma. A cell culture portion and an in vivo mouse cortex experiment were utilized to analyze the changes in the microvasculature network and the activation of the surrounding microglia related to two specific secretion factors in combination with the BRAFV600E mutation.

Introduction

Low grade glioma (LGG) can be characterized as a brain tumor, formed from either of two distinct types of neuroglia, astrocytes or oligodendrocytes, transformed into tumor cells by somatic mutations. These tumors often are identified in patients by epileptic seizures. LGG are classified as grade 2 tumors, the slowest growing classification of brain tumors [1]. Like many other cancers, these tumors often exhibit mutations in the v-raf murine sarcoma viral oncogene homolog B1 (BRAF) [2]. More specifically, the BRAFV600E mutation, where the 600th amino acid in the sequence is mutated from a valine (V) to glutamate (E), caused by aberrant activation of v-raf signaling. These mutations are common amongst low grade gliomas, up to 60% in pediatric pilocytic astrocytoma, yet are very rare in more severe brain cancers such as adult

glioblastoma multiforme (Grade IV) or anaplastic astrocytoma (Grade III), which are 1-2% and 2-5% respectively positive for BRAFV600E [2]. In addition to tumor cells displaying aberrant growth, they also secrete factors that reorganize the tissue surrounding them. This reorganization specifically includes changes in vasculature and the increased activation of microglial cells which may cause neurological deficits associated with LLG. Two secreted factors that will be the focus of my project, GDF-15 and CCL-3, are released by cancer cells [3], and were identified in preliminary data in the LoTurco lab to be upregulated in expression by BRAFV600E mutations in astrocytes. The LoTurco Lab has determined an animal model needed to investigate the formation of these tumors in a mouse model.

The hypothesis investigated this summer while in the LoTurco lab was that increased expression and release of GDF-15 and CCL-3 in astrocytes that carry BRAFV600E will cause a change in vasculature and activation of microglial cells around those cells. More specifically, we aimed to assess whether the factors can affect the microglia, macrophage-like cells of the brain, and the growth of vasculature in the developing brain [4].

Materials and Methods

Experimentation was performed in two distinct stages: an astrocyte cell culture portion and an in vivo experiment in a mouse model of LGG. To make the CRISPR targeting plasmids, I utilized the molecular biology design tool, Benchling, to design CRISPR/Cas9 plasmid DNA vectors needed to target GDF-15 and CCL-3 genes in astrocytes and in the BRAFV600E LGG mouse model. The CRISPR constructs were made by annealing, ligating DNA, and transforming bacteria. Following this, single bacterial colonies and purified plasmid DNA were grown in culture. These DNA plasmids were then sent out for DNA sequencing to confirm that they

contained the CRISPR/CAS9 knockouts for GDF-15 and CCL-3 with the BRAFV600E mutation. After confirming that the DNA constructs were correct, larger amounts of the plasmid were created in order to concentrate before the in vivo portion of the experiment. This stage resulted in three failed, insufficient plasmid concentrations through a maxi prepping procedure before a fourth yielded the necessary concentration needed for the mice surgeries with the aid of an ethanol precipitation.

There were four distinct groups of astrocytic cell groups prepared for the surgeries on the mice: a control group transfected to express Green Fluorescent Protein, one with the BRAFV600E transgene, one with a CRISPR/Cas9 plasmid targeting construct against GDF-15with BRAFV600E, and one with a CRISPR/Cas9 targeting construct for CCL-3 withBRAFV600E. These cultures were repeated in triplet. After a three-day culture period, the cells were harvested and processed for RNAseq to confirm the effectiveness of CRISPR/Cas9targeting of CCL-3 and GDF-15 and any off target effects. Genomic PCR and sequencing were additionally utilized to ensure that indels, deletions or small genetic insertions, were created in the GDF-15 and CCL-3 genes by the CRISPR/Cas9 plasmids.

In the in vivo experiments, the same CRISPR and BRAFV600E conditions described above for the cell culture experiments were used in the developing mouse cortex in vivo. The In Utero Electroporation (IUE) technique was utilized, and performed by Graduate Student Kevin Truong, to insert the plasmid DNA into the embryo neural cortex with the help of electrical pulses to force some of the neural cells to accept the DNA, resulting in genetic alteration. Three weeks after the mice were born and the pups no longer required their mother’s milk, the mice brains were perfused to fix the brains for the purpose of preserving the neurological proteins and neural structure. The brains were then sliced to a sixty nanometer thickness to be prepared for an

immunohistochemical stain. This process utilizes primary and secondary antibodies linked to specific fluorescent wavelengths to then be seen optimally under a fluorescent microscope. The IBA-1 biomarker for microglial activation while the CD-31 biomarker was utilized to mark microvasculature activation. The number of IBA-1 and CD-31 positive cells in proximity to BRAFV600E expressing regions are in the process of being quantified for each of four brains in each condition. A confocal microscope was used to capture higher resolution and more focused images of structures such as heterotopia, the parenchyma, and the pial surface. A fluorescent macroscope was used to analyze individual hemispheres of brain tissues for the purpose of comparing transfected and transfected portioned of brain tissue on a larger scale. Both microscopes were able to confirm the effectiveness of the antibodies stained during immunohistochemistry. In order to analyze the percent of space covered by vasculature in the regions expressing BRAFV600E in the different conditions will be compared to determine whether blood vessels growth is altered by deletion of CCL3 or GDF15 through a software called ImageJ/Fiji. To identify the extent of the microglial activation within the transfected portions of the brain, we will be using the ImageJ/Fiji software to count the activated cells over a specific area of tissue.

Materials needed for my experimentation were provided by the LoTurco lab, including the mice specimen, cultures, specific genes, RNAseq access, and genomic PCR were covered by an NIH grant: Developmental Pathophysiology of Somatic Mutations in Developing Neocortex.

Results

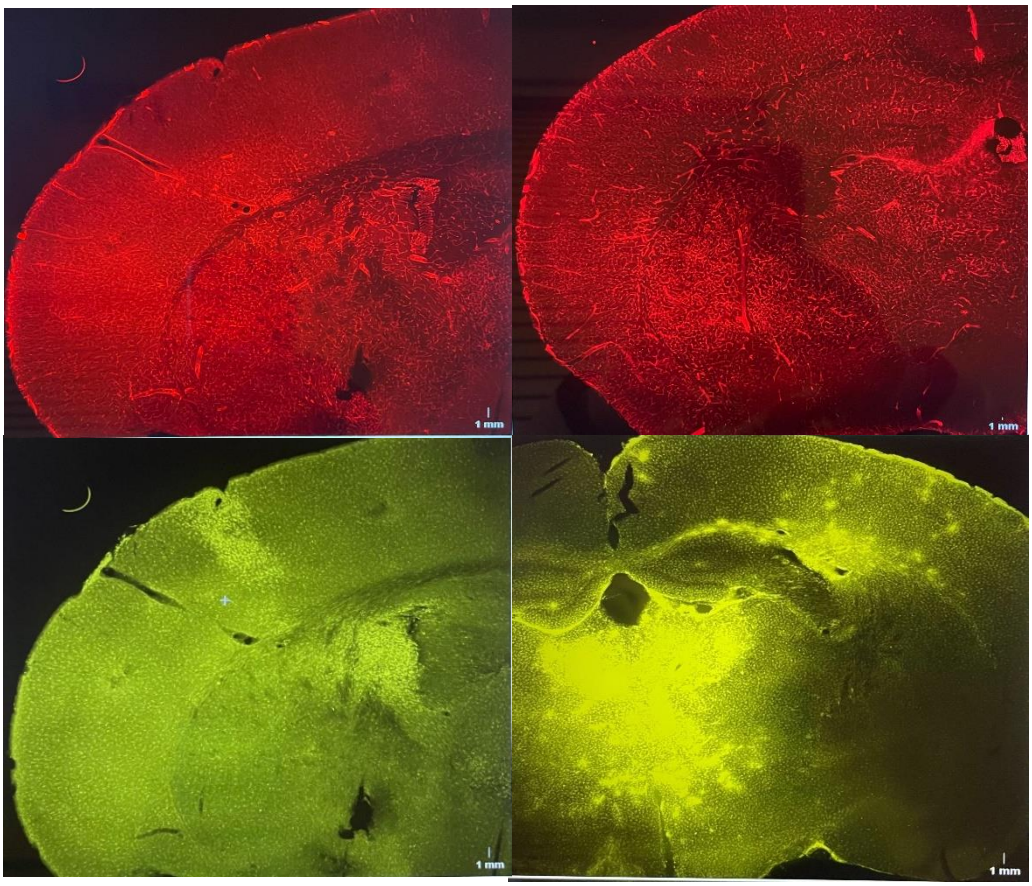

Figure 1: **Analysis of CD-31 and IBA-1 Transfection in Mouse Neural Cortex's with GDF-15 and CCL-3 CRISPR/Cas9 Knockouts** a. The top left image is representative of GDF-15 transfection with the CD-31 addition to highlight microvasculature alteration. b. The top right image is representative of the CCL-3 transfection with the CD-31 addition to highlight microvasculature alteration. c. The bottom left image is representative of the GDF-15 transfection with IBA-1 addition to highlight the microglial activation. D. The bottom right image is representative of the CCL-3 transfection with IBA-1 addition to highlight the microglial activation.

In preliminary assessment of my tissue, not yet fully quantified, I have observed activation of the microglia in the GDF-15 experimental condition, and what appears to be more activation in the CCL-3 condition in comparison. The difference lies in the medial portion of the CCL-3 conditions, with increased activation compared to a significantly lower rate of transfection with the GDF-15. In terms of changes to the microvascular network of the neural tissue, there was significant microvascular growth in certain regions of the GDF-15 specimen in regions where BRAFV600E expressing neurons formed misplaced groups of cells or heterotopia, but this pattern of increased vascularization was less apparent in the CCl3 treated brains, especially along the lining of the major neural blood vessels.

Discussion

The results collected so far should be considered completely preliminary but with the potential to yield meaningful results following a full analysis. I will need to quantitatively analyze the staining patterns I have obtained in these conditions and increase the number of brains collected and processed in each group. For this analysis I am using a software package called ImageJ/Fiji to quantify the density of the microvascular network in the three conditions, as well as assess the intensity of microglial staining to determine microglial activation. At this point we are unable to accept or reject the starting hypothesis, but have the reagents needed, and technical knowledge now, to complete the experiment, and determine whether the genes encoding the secreting factors CCL3 and GDF15 are involved in the neurological pathologies associated with BRAFV600E mutations.

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