Mutant Epidermal Growth Factor Receptor “Velvet” Model in Astrocytes and its Role in Glioma Progresssion

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Introduction:

Glioblastoma multiforme is the most proliferative and invasive of primary brain tumors making them essentially incurable, as recurrence can occur even with 98% of the tumor cells removed . It is well known that the overactivation of epidermal growth factor receptor (EGFR) is associated with glioma progression and proliferation. However little is known about the role of EGFR activation in the local environment in response to the tumor. We hypothesize that activation of EGFR in progenitor cells and astrocytes near the site of an advancing glioma contributes to the progression of invading glioma in the brain.

To determine the role of EGFR in the brain’s response to glioma invasion, we used mutant “velvet” mice with a genetic mutation of the conserved DFG motif of the tyrosine kinase domain, preventing the EGF receptor from autophosphorylating but keeping the quarternary structure intact. As such it is a dominant mutation that can disrupt the signaling of the normal allele. Mice with the heterozygous dominant mutation are identifiable by their abnormal wavy (velvet) hair and their partially open eyelids at birth[1].

We set out with two goals:

1. To determine the in vitro physiological difference of EGFR-mutant astrocyte cultures in response to EGFR ligands and challenged by invasive or non-invasive glioma cell lines.
2. To determine in vivo if the EGFR-inactive microenvironment of the “velvet” mice has an effect on glioma progression through the brain.

Models and Methods

In Vitro Methods

Astrocyte Isolation

Brains were isolated from mouse pups less than three days old. The cortex was removed of any meninges under dissecting microscope, minced, and then incubated with collagenase and trypsin . The cells were then re-suspended in their 1:1 Astrocytic DMEM media. This process selected for a glial culture composed of astrocytes.

Proliferation Assay

Cells were seeded to a 196-well plate at a concentration of 4,000 cells/well (100 ul/well) and incubated overnight in growth media at 37ºC. Velvet and WT cells were plated with the following conditions: 1:1 basal media with and without TGF treatment, and growth media with and without TGF treatment. The next day the media was exchanged for new starvation basal media and growth media conditions. TGF treatment was added to the cells at a concentration of 10 ng/ml 6-7 hours after the new starvation basal media and growth media were added (Day 0). Cells were stained at days 1, 3, and 5 using sulforhodamine B based assay and the cell density was measured at 490 nm.

Migration Assay

Velvet and WT astrocyte cells and GL261 tumor cells were dissociated and seeded into cell culture inserts at 100,000 cells per insert overnight in cell culture media. Either Velvet or WT astrocytes were plated in one chamber while GL261 tumor cells were plated in the other. A barrier in the culture insert creates a gap between the two individual cell patches much like a scratch assay. The following day the culture inserts were removed and the media was replaced with different conditions of growth media, starvation media, and starvation + TGF-α treatment (10 ng/ml). Pictures of the gap between the astrocyte and tumor cells were taken at days 1, 2, and 3. The unoccupied space in the gap was then measured using imageJ MSV gap assay software and plotted using Graphpad Prism.

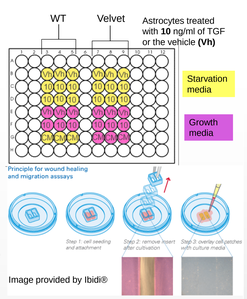
In Vivo Methods

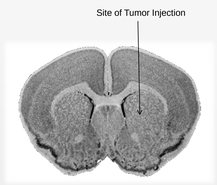
Glioma Cells

The Glioma 261 cell line (GL261) was kindly provided by Dr. Mariano Viapiano (Dept. Neurological Surgery, OSU), with permission from Dr. Xandra Breakfield (Massachussets General Hospital). The M4 cells were kindly provided by Dr. Chang-Hyuk Kwon (Dept. of Neurological Surgery, OSU). Gl261 cells were stably transfected with eGFP cDNA and M4 cells with ds2RedFP cDNA.

Intracranial Microinjection

All procedures involving mice were conducted in accordance with the National Institutes of Health regulations concerning the use and care of experimental animals. All studies involving animals were approved by the institutional Animal Care and Use Committee at The Ohio State University (OSU IACUC 2009A0165)
Glioma cells were harvested and counted using a hemocytometer. Glioma cells were then re-suspended at **25,000 cells/ul for M4 cells** or **50,000 cells/ul for Gl261 cells**. A syringe was inserted into the mouse striatum at a depth of 3.5 mm, 0.5 mm rostral to the bregma, and 2 mm lateral of the midline in the right hemisphere. The glioma suspension was injected with 2 ul of glioma cell suspension for a total of 50,000 M4 cells or 100,000 GL261 cells. Staples were removed one week post-surgery. Mice showed no difference in behavior over the three weeks. After the three weeks the mice were sacrificed and the brains were examined for tumor growth using IHC.





In Vitro Results

Reduced Activation of EGFR and Down-Stream MAPK Signaling

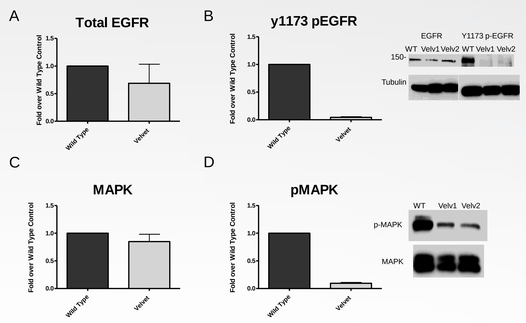


Fig. 1
Western blot of Wild Type and mutant Velvet (n=2) astrocytes grown in culturing media in the presence of serum. EGFR signals its activation pathway by autophosphorylation. A lack of phosphorylated EGFR will confirm that the mutant mice have knocked-out EGFR activation and that the receptor can no longer autophosphorylate. **(A)** There is no significant difference in total EGFR expression. **(B)** Velvet astrocytes have a 25-fold decrease of EGFR phosphorylated at the y1173 tyrosine residue.

Mitogen-activated protein kinase (MAPK) is a downstream target of the EGFR pathway. An absence of phosphorylated MAPK can represent a lack of upstream EGFR signaling. **(C)** There is no significant difference in MAPK expression. **(D)** Velvet astrocytes have a 10-fold decrease in phosphorylated MAPK.

Velvet Astrocytes do not Proliferate in Response to TGF-α Treatment

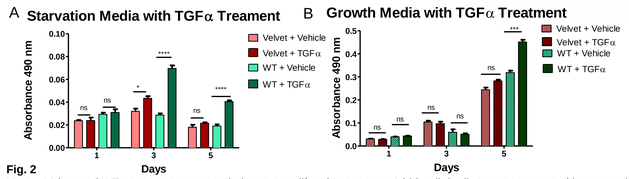


Fig. 2
Mutant Velvet and WT astrocytes were seeded onto a proliferation assay at 4,000 cells/well. Astrocytes were either treated with TGF-α (10 ng/ml) or with the vehicle (0 ng/ml). **(A)** Astrocytes were grown in starvation conditions with or without the TGF treatment. WT astrocytes demonstrate a significant response to the TGF-α stimulation. Velvet astrocytes also show a less significant response to TGF-α at day 3. **(B)** Astrocytes were grown in culture media in the presence of serum with or without TGF treatment. There is no real significant difference in growth until day 5; the wells have reached confluence by then as well.

Velvet Astrocytes Do Not Migrate in Response to TGF-α Treatment

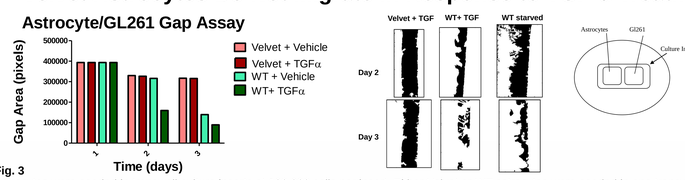


Fig. 3
Astrocytes were seeded in transwell culture inserts at 100,000 cells per insert. Either Velvet or WT astrocytes were seeded in one transwell while Gl261 glioma cells were seeded in the other. The cells reached adhered to the plate overnight and the culture insert was removed the following day. Pictures of the gaps between the astrocytes and GL261 were taken over days 1-3. The area of the gap between the astrocytes and the tumor cells where no cells exist was then calculated, **the lower the gap represents the greater the migration of the astrocytes over time**. A significant decrease in the gap area and an apparent migration of astrocytes is observed for WT cells, with and without TGF-α treatment. There is minimal migration of theVelvet astrocytes.

Velvet Astroyctes Don’t Show Bipolar Morpholoy with TGF-αTreatment

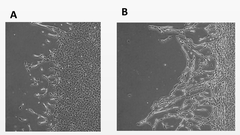


Fig. 4
Pictures of Velvet and WT astrocytes after 6 days of TGF-α treatment in starvation conditions. (A) Velvet astrocytes show a stellar morphology representative of mature astrocytes. (B) WT astrocytes show an elongated bipolar radial morphology reminiscent of early developmental neural stem cell morphology.

ErbB Family/PDGFRA Gene Expression in Velvet Astrocytes

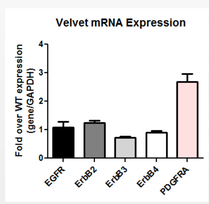


Fig. 5
To determine if EGFR inactivation was compensated by increased expression of the other ErbB family members or by platelet-derived growth factor receptor (PDGFRA), reverse-transcriptase PCR was used to measure gene levels in mutant astrocytes cultures grown in the presence of serum. Analysis of the expression of mutant astrocytes relative to WT astrocytes expression shows no significant difference in EGFR, ErbB1, ErbB2 or ErbB3. Analysis shows around a 3-fold increase in PDGFRA expression in mutant astrocytes relative to WT astrocytes cells.

In Vivo Results

No Quantitative Difference in Volume of Non-Invasive Tumor

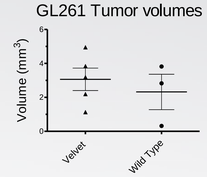


Fig. 6
Green fluorescent protein expressing GL261 tumor cells were injected intracranially (100k cells/brain) into mutant velvet (n=5) and WT mice (n=3). Three weeks later the brains were perfused, cryosectioned, and measured for tumor volume. Using the Cavelieri method, the volume of the tumors was estimated across n > 20 sections per brain. Analysis reveals a highly variable tumor size with no significant difference of tumor volume between the velvet and WT brains.

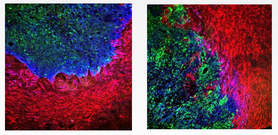


Fig. 7
IHC stains of WT and Velvet brains. Representative images show GFAP reactive astrocytes (red) surrounding the tumor to form a glial border. Nestin (blue) marks proliferating and migrating cells. No qualitative difference has been observed in the astrocytic response or the invasiveness of the tumors between the normal and mutant brains.

Current Work on Invasive Tumor Model

With little difference observed in the Gl261 glioma model, we looked for a more invasive tumor model to challenge the mutant astrocytes. Similar-sized neurospheres were taken from the M4 glioma cell line and placed individually on laminin-coated plastic plates. The M4 cells on the surface were observed to have quickly migrated. Red fluorescent protein expressing M4 tumor cells were injected intracranially (50k cells/brain) into mutant velvet (n=8) and WT mice (n=9). Three weeks later the brains were cryosectioned. Initial observation showed that the M4 red fluorescent cells were highly invasive and were seen migrating through the white matter. The M4 core tumor volume was highly variable between the injections.

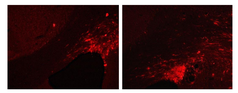


Fig. 8
Representative images of RFP-expressing M4 glioma cells (red) migrating into the corpus callosum from the right hemisphere. This is a clear indication of an invasive tumor model.

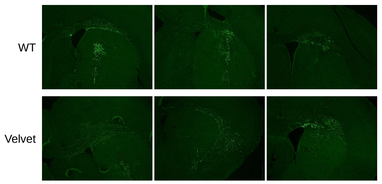


Fig. 9
Representative images of (green) Ki-67 expressing cells. This is indicative of cells that are undergoing cell division and are proliferating. We observed proliferation at the site of the tumor.

Discussion

The in vitro data confirms what we have seen in the current literature[1,2]. It was shown that EGFR tyrosine phosphorylation by EGF stimulation is inhibited in vivo by the lab that induced the mutation. The impairment of proliferation and migration in mutant astrocytes also confirms what we would expect in vitro. To further show that EGFR inactivation results in a loss of migration in the astrocytes, we can use antibodies against Rho/Rac and proteins responsible for actin reorganization such as p-cofilin, p-ezrin, and p-VASP

We have also found that normal astrocytes in starvation conditions will still migrate and form a radial morphology in the presence of a glioma tumor. Velvet mutant astrocytes with impaired EGFR autophosphorylation do not show this response in starvation conditions. This suggests that the astrocytes are responding to an EGF or TGF-α autocrine loop by the glioma cells. The radial morphology of the astrocytes when stimulated by TGF-α reflects an early developmental phenotype of neural stem cells. This morphology may form a more plastic environment that allows for the tumor invasion.

The variability of Gl261 and M4 gliomas in tumor volume pose to be difficult to our in vivo study. The Gl261 gliomas were non-invasive however, so the tumor model was more representative of a systemic metastatic tumor that had entered the brain rather than a primary glioma tumor. The M4 tumor represents a much better model, we observed diffuse invasion into the surrounding brain tissue, similar to a human GBM. However no conclusive data has been collected from the in vivo M4 study. To find a significant impairment of glioma invasion with mutant astrocytes, an intensive study of specific regions of the brain and glioma will have to be done.

The data obtained from this project is being replicated through collaboration with Dr. Viapiano’s lab. Preliminary results from that lab suggest a significant increase of M4 glioma cell migration on mutant brain slice assays. We also now have a working invasive glioma cell line that can be used. This is a positive step towards a working in vivo model of the effect the mutant astrocytes have on the invasion of the tumor.

References:

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2. White RE, Rao M, Gensel JC, McTigue DM, Kaspar BK, Jakeman LB. Transforming growth factor α transforms astrocytes to a growth-supportive phenotype after spinal cord injury. J Neurosci. 2011 Oct 19;31(42):15173-87.

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