Slice Culture

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I. Objective

To use sub millimeter brain tissue sections obtained from a rat as a model to represent white and gray matter as well as components of the extracellular matrix in the brain. To visualize migration of F98 rat-derived glioblastoma cells into tissue sections using immunohistochemical staining.

II. Abstract

Glioblastoma multiforme (GBM) is the most aggressive form of astrocytoma and accounts for the majority of primary malignant brain tumors among adults in the United States (Ostrom et al. 2014). Evidence shows that sulfated CS-GAGs may play a role in tumor invasion, but the driving conditions and role of the brain environment in glioma invasion are currently unknown (Logun 2016). A method called "slice culture" was created in order to investigate these CS-GAGs. Slices from explanted rat brains were taken in 50 μm sections on charged slides, biopsy punched, and then overlaid with glioma cells in agarose hydrogels. After 48 hours, migration and spread was observed and tissue was fixed for immunohistochemistry. Antibody staining labeled cells for glioma stem cell markers using CD133 and for cells expressing CS-GAGs using CS56. High co-localization was observed for cells expressing CD133 co-localized with CS59 as well as for cells expressing CS59 co-localized with CD133. These results demonstrate that slice culture is a successful model to look at the brain and tumor microenvironment and visualize cell type, migration, and spread.

III. Introduction

Brain tumors are among the most destructive malignant cancers and account for approximately 3% of new cancer cases (Ostrom et al. 2014). One-third of brain tumors originate from support cells called glial cells, giving the tumor the name "glioma" (Ostrom et al. 2014).

Astrocytic gliomas, also called “astrocytomas” develop from astrocytes which are star-shaped cells that play a role in connective tissue and the blood-brain barrier (Huttner 2012; Marquet et al. 2007; Romeike 2007).

The most aggressive, malignant astrocytoma is glioblastoma multiforme (GBM) which is a grade IV glioma and accounts for the majority of malignant gliomas in the United States (textbook ch; Ostrom et al. 2014). Grade IV is the highest grade and indicates a high degree of abnormality in tissue and a high rate of growth of the tumor. Grade IV GBM is characterized by the presence of necrosis and blood vessel formation around the tumor, distinguishing it from grade I-III gliomas (Castillo et al. 2014). Glioma cells are known spread through the brain along a path called “Scherer’s structures” in which the glioma cells travel from brain parenchyma to blood vessels and white matter tracts, and eventually enter the sub-arachnoid space in the brain meninges (Cuddapah et al. 2014). The spread of the glioma cells throughout essential brain regions culminates in multiple GBM masses (Batzdorf and Malamud 1963).

GBM is unique in that it does not leave the CNS and metastasize to other areas of the body, but rather is confined to the CNS. Its growth in such a small, limited space eventually leads to damage of vital brain regions resulting in impairment and loss cognitive and motor processes (Kleihues and Sobin 2000). The current standard of care treatment is complete surgical resection; however, it has been shown to have no effect on the survival of the patient who will succumb to the tumor within a few months to a year even with the best possible surgical procedure and follow-up care (Jelsma and Bucy 1967). Complete surgical resection is inadequate for treatment because it cannot stop the cells that have already begun migrating from the tumor mass into other areas of the brain. Non-uniform boundaries and heterogeneity of the tumor also contribute to resection failure. Radiation therapy and chemotherapy can be used, but tumor

recurrence is common with treatment or resection (Wakimoto et al. 2009). There are no therapies that target glioma cell invasion, and the driving conditions and role of the brain environment in glioma invasion are currently unknown.

Abnormal conditions of components of the extracellular matrix in the brain are linked to and can directly promote cancer cell migration (Cox and Erler 2011). Healthy brain parenchyma contains chondroitin sulfate proteoglycans (CSPGs), hyaluronic acid, laminin, collagen IV, and fibronectin. The CSPGs have chondroitin sulfate glycosaminoglycans side chains (CS-GAGs) bound to their core protein (Lau et al. 2013). The CS-GAGs linked to CSPGs are known to bind and organize the brain extracellular matrix as well as regulate neuronal outgrowth (Ruoslahti 1996). The GAGs have different sulfation patterns; chondroitin-4-sulfated GAGs (CS-A) make up the majority of GAGs in a healthy brain, and there are smaller percentages of chondroitin-6-sulfated GAGs (CS-C) and chondroitin-4,6-sulfated GAGs (CS-E) (Sugahara and Mikami 2007). Upregulation of CSPGs, specifically the di-sulfated CS-E GAG units, is known to occur around brain tumors, but the role of sulfated CS-GAGs in promoting glioma invasion is unknown. Since the sulfated CS-GAGs appear in abundance in the tumor periphery area and are able to bind cell motility and adhesion factors, it is probable that there is a CS-GAG sulfation-driven mechanism that plays a role in tumor invasion.

GBM is highly heterogeneous and expresses inconsistent antigenic signals and growth rates as well as genomic variability throughout the tumor mass (Bonavia et al. 2011; Furnari et al. 2007; Yung et al. 1982). Glioma stem cells, which are similar to normal stem cells, may be the cause of the heterogeneity (Clarke and Fuller 2006). Glioma stem cell theory states that there is a population of tumor cells that is directly responsible for self-renewal and tumorigenesis (Visvader and Lindeman 2008). Glioma stem cells express the cell-membrane protein CD133

like neural stem cells (Singh et al. 2003, 2004). CD133 is a glycoprotein in cellular protrusion that has been associated with certain cancer cells (Corbeil et al. 2000; Horn et al. 1999; Sanai et al. 2005; Singh et al. 2003). The glioma stem cells are able to survive by remodeling the ECM and vasculature so that they can self-renew without other factors or nutrients (Li and Neaves 2006).

Modeling brain tumor invasion is a challenging task. While *in vivo* models can provide living, 3D environments for the tumor to grow, the viability of the animal presents a challenge when monitoring the progression of the tumor. 2D surfaces can also be useful in some respects, but lack in the ability to assess cell-cell interaction or cell response to extracellular cues which need to be investigated (Lovitt, C. J. et al. 2014). The most commonly used models currently are 3D models which have tumor spheroids encased into a designed polymer scaffold or hydrogel and allow for study of cell-ECM interactions (Charoen et al. 2014). 3D cultures are necessary in order to replicate cellular heterogeneity and multicellular structure which play a role in cancer mechanisms (Loessner et al. 2010)

A 3D model called “slice culture” was created. Slice culture consists of slices derived from sub millimeter brain tissue sections which are used as a way to look at the full multicomponent brain environment, including white and gray matter, and components of the ECM such as CSPGs, CS-GAGs, hyaluronic acid, laminin, collagen IV, and fibronectin. Rather than use a model of solely the hippocampal area, which is a common model, the entire slice of brain tissue is used in order to visualize multiple components of the extracellular matrix matter for the most realistic representation of the brain environment that would be present during the growth of a tumor. The slice culture model allows for visualization of the growth and spread of a

tumor throughout the brain. The model satisfies the need for a 3D structure that replicates cellular heterogeneity and multicellular structure.

In the model, the sectioned brain tissue is biopsy punched and then overlaid with a hydrogel containing F98 cells. F98 cells are rat-derived glioblastoma cells that are cultured for use in the model. The F98 cells are observed to migrate into brain tissue over 48 hours and then immunohistochemical staining is used for further visualization and analysis. The model is easily repeated since it uses brain tissue sections rather than the entire brain. Slice culture provides visualization of tumor invasion *in vitro* and displays the kind of environment that glioma cells are drawn to as well as what areas they localize in.

IV. Materials and Methods

Preparation and Processing of Brain Tissue

Animals were transcardially perfused with 4% paraformaldehyde in 1X PBS. The brains were snap frozen with dry ice and stored at -80° C. Brain tissue was sectioned at a thickness of 50 μm using a Leica CM1950 cryostat set at -20° C, and serial sections were collected on charged glass slides (VWR, PA). A .5 mm biopsy punch was taken from directly below the left side of the corpus callosum. The slides were then left at -20° C before further use.

Cell Culture

F98 rat-derived glioblastoma cells from ATTC were cultured in media consisting of DMEM/F12 (Corning, NY) and 10% Fetal Bovine Serum (Corning, NY); and under 37° C and 5% CO_2 incubation. Cells were fed with supplemented media every other day unless passaged or extracted for other use.

Slice Culture Procedure

Agarose hydrogel suspensions were fabricated using a mixture of DMEM/F-12 (Corning, NY), SeaPlaque Agarose, and F98 cells. The hydrogel suspensions were prepared with 0.5% agarose in DMEM/F-12. The F98 cells were re-suspended at a concentration of 20,000 cells per 1μL DMEM/F12. 2 μL of the hydrogen suspension was plated into the biopsy punched area. The plates were then overlaid with slice media. The slice media consisted of standard medium of DMEM/F-12 at a pH of 7.3 (Corning, NY), 1% Penicillin Streptomycin, 24% Fetal Bovine Serum (Corning, NY), and 10mM D-glucose (St. Louis, MO). At 48 hours, they were fixed with 4% paraformaldehyde for 30 minutes and then either left in PBS or used for immunohistochemical analysis.

Immunohistochemistry

Two-part antibody staining was performed on the slice cultures. The slices were incubated in penetration buffer consisting of PBS with 0.2% Triton (St. Louis, MO) with 0.3M glycine (Fair Lawn, NJ), and 20% dimethyl sulfoxide (St. Louis, MO) at 37° C for two hours with gentle shaking. Then the samples were blocked in blocking buffer consisting of PBS with 0.2% Triton, 6% goat serum (LOCATION), and 10% dimethyl sulfoxide at 37° C for one hour. Samples were then washed with 1X washing buffer for 1 hour, twice. Washing buffer consisted of 10X PBS with 0.2% Tween-20 (Fair Lawn, NJ) and 10 μg/mL heparin (St. Louis, MO) diluted in PBS. Samples were transferred to primary antibody dilutions in the antibody buffer and incubated at 37° C with gentle shaking overnight. The samples were then washed in washing buffer 10 times for 10 minutes each, each with gentle shaking. The samples were then incubated in antibody buffer at 37° C with gentle shaking overnight. DAPI Nuclear stain was added in washing buffer within was secondary antibody solution and then the samples were incubated at 37° C for two hours with gentle shaking. Then the samples were then washed in washing buffer

10 times for 10 minutes each, each with gentle shaking. Finally, Fluoromount was used to preserve the slides and they were overlaid with glass cover slips before imaging.

V. Results

In order to visualize cell migration and spread of F98 cells through the slice culture model, tiled images were taken. The slice cultures were antibody stained with three different markers. Hoechst stain marked DNA, CD133 marked stem cells, and CS59 marked CS-GAG expression.

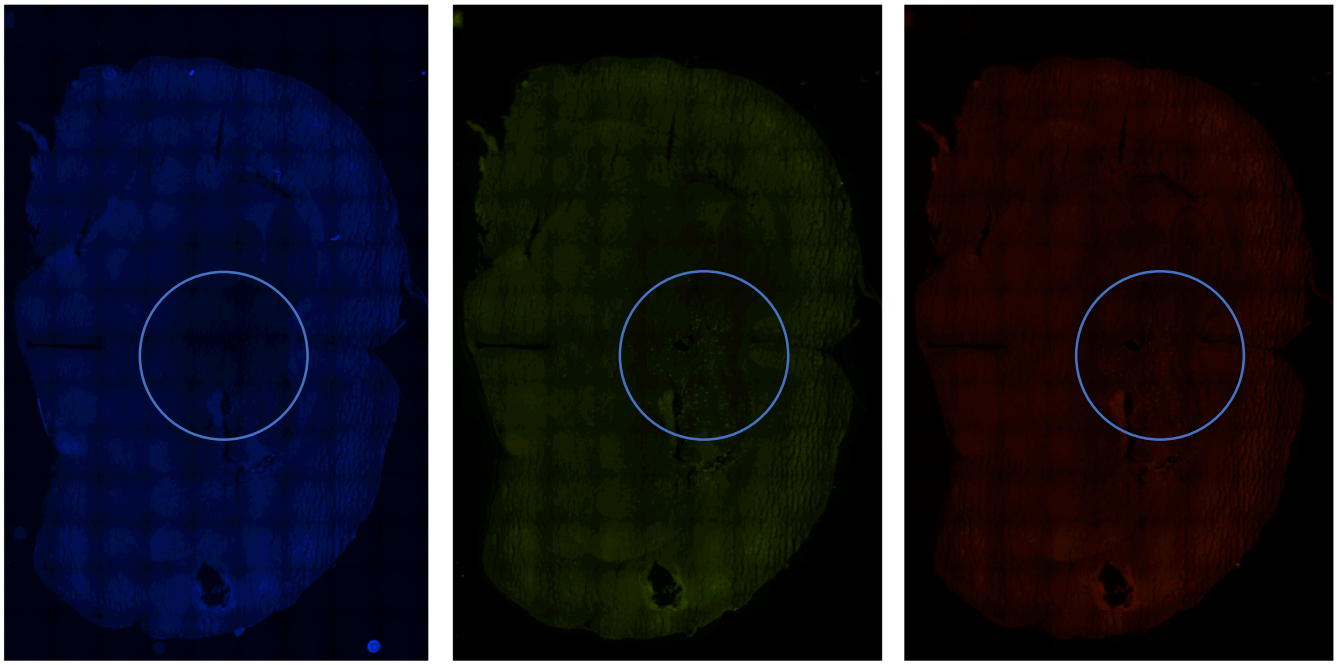

Figure 1: 10x full slice images stained for Hoechst (blue), CD133 (green), CS56 (red)

In the tiled images, the entire slice as well as the hippocampal area can be seen (Figure 1). The agarose droplet was placed under the left side of the hippocampus, so it is clear that the cells have migrated and spread out from this region. Some of the cells are seemingly accumulating towards the sub-ventricular zone (SVZ) and along the corpus callosum.

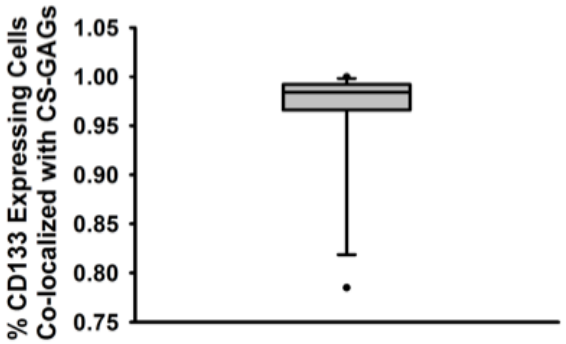

Figure 2: Co-localization measured using Velocity to visualize antibody stained 50 µL sections. CD133 expressing cells co-localized with CS56 expressing cells.

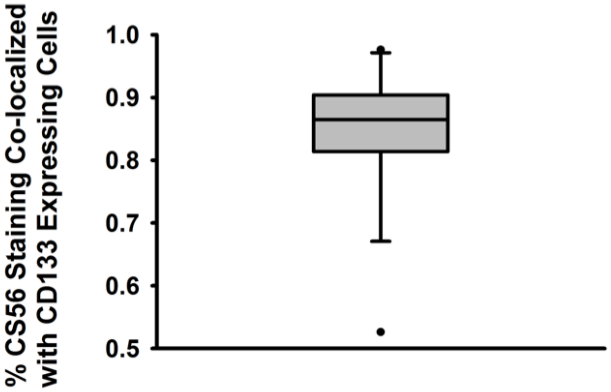

Figure 3: Co-localization measured using Velocity to visualize antibody stained 50 µL sections. CS59 expressing cells co-localized with CD133 expressing cells.

10x images were then taken of randomized areas of the slices in order to measure co-localization of the stains. Co-localization was measured for cells expressing CD133 co-localized with CS59 (Figure 2) as well as for cells expressing CS59 co-localized with CD133 (Figure 3). Cells expressing CD133, the stem cell marker, had a high amount of co-localization with cells which also expressed CS59, the CS-GAG marker (Figure 2). High co-localization is also supported by the third quartile of the box and whisker plot because most of the values are near the median. Cells expressing CS56 were highly co-localized with cells which also expressed CD133 (Figure 3).

VI. Discussion

Glioma cell invasion poses the biggest obstacle to GBM treatment. Although surgical resection, chemotherapy, and radiotherapy may be used, they are incapable of stopping glioma cells that migrate from the tumor mass into other areas of the brain. Our results show that there is likely an ECM component of GBM which directly promotes glioma cell invasion. In this study, the slice culture model was created in order to study glioma invasion with 3D architecture and

ECM components represented in the most realistic way without being *in vivo*. The model was successful as it allowed for visualization of F98 glioma cell invasion into a tissue sample. The 50 μm slices modeled the white and gray matter of the brain as well as other ECM components we were interested in investigating such as CSPGs, CS-GAGs, hyaluronic acid, laminin, collagen IV, and fibronectin. Cell migration was visualized based on comparison of the location of the agarose droplet with the location of the cells after 48 hours. Our model allowed for antibody staining using pre-established laboratory procedures. Typically, antibody staining of entire tissue sections proves to be difficult because of antibody penetration and blocking. However, our results showed that our slice culture model is compatible with our antibody staining procedure and means that cells will not need to be pre-labeled in further experiments using slice culture. Our staining procedure labeled cells for glioma stem cell markers using CD133 and for cells expressing CS-GAGs using CS56.

In the tiled images, cells were observed to accumulate in and around the sub-ventricular zone (SVZ). The SVZ is a neural stem cell niche. The SVZ is also known to be an area containing many CS-GAGs (Singh 2003). The spread to this area supports the glioma stem cell hypothesis that stem capability may be endogenous to GBM tumors. High co-localization was observed for cells expressing CD133 co-localized with CS59 as well as for cells expressing CS59 co-localized with CD133. Since CS-GAGs and glioma stem cells have such high co-localization, it is clear that they both have a role in glioma invasion, and are likely endogenous to glioma tumor growth. The high co-localization is also relevant because it shows that the tumor cells are moving towards GAG rich areas. Since GAGs bind and retain growth factors, when the stem cells move into the SVZ area they could be secreting more GAGs and in turn promoting

differentiation. The GAGs in the SVZ could also be enhancing the ability of the glioma stem cells to stay in the neural stem cell state.

The niche of the tumor itself is also important to investigate because the tumor ECM contributes to the breakdown of normal cell signaling and ECM dynamics (Lu et al. 2012). Certain ECM components are shown to be overexpressed in cancerous tissue, such as sulfated CS-GAGs and CD133, which likely contributes to increased tumor growth. Upregulation of specifically the di-sulfated CS-E GAG units is known to occur around brain tumors, but the role of sulfated CS-GAGs in promoting glioma invasion is unknown. In the tiled images, CS56 labeled cells expressing CS-GAGs are observed. Since the sulfated CS-GAGs appear in abundance in the tumor periphery area, and are able to bind cell motility and adhesion factors, it is probable that there is a CS-GAG sulfation-driven mechanism that plays a role in tumor invasion.

Cells that express high levels of CD133 are shown to contribute to radioresistance because they increase the cellular repair mechanisms for DNA damage (Bao et al. 2006a). When these CD133 expressing cells remain after radiation and chemotherapy have targeted the larger tumor mass, they have increased resistance capabilities which is likely part of why GBM has such a high rate of recurrence.

Though the slice culture model was able to provide a platform in which cell migration could be measured among components of the extracellular matrix which we were interested in, several limitations to the model exist. The main limitation to the procedure was that several of the sub-milimeter slices began to flake off when washed with buffer. The slices that flaked off were not adhered strongly enough to the charged slides to withstand the force of liquid washing over them. In the future, this limitation could possibly be overcome by using thicker brain slices

which would adhere stronger to the charged slides. Another limitation to the model is that, although it does provide a platform to visualize many brain components, it is still only a small fraction of the total brain volume. However, it would be impractical and inefficient to use an entire brain to investigate F98 cell migration. If an entire brain were used, MRI scanning would have to be used rather than microscopy imaging, which would be time-consuming and likely not provide good visualization of migration. The slice culture model is further limited in that the area under the left side of the corpus callosum has to be biopsy punched in order to provide a space of F98 cells to be inserted. This is a limitation because that entire area is removed, so if it played a role in tumor invasion, we would be unable to visualize it. This leads to another limitation which is that by introducing cells *in vitro*, we are unable to visualize the true beginning of tumor investigation, we can only investigate migration, spread, and cell expression. In the future, it would be beneficial to alter the model in a way that we could also investigate the initial growth of the tumor in some way, rather than only investigating its effects once it is present in the brain.

In the future, visualization of cell migration could be improved by using agarose droplets doubled in size containing more F98 cells. Also, live cell imaging could be used in order to visualize the pathway that the cells followed during initial invasion, rather than simply analyzing their start and end points. Live cell imaging would contribute data about which structures the cells moved through as they invaded the brain, which could then be further investigated in order to discover the actual mechanism by which they propagate throughout the brain. Live cell imaging could also contribute information regarding potential invasive sub-cell populations and their different rates of growth. Investigation of sub-cell populations could potentially lead to more treatment options for GBM patients, because elimination of sub-cell populations would allow efforts to be focused on removal of the main, large tumor mass.

The slice could also be treated with a GAG antagonist such as Surfen. Surfen is a small molecule that has demonstrated an ability to block sulfation and degradation of GAG chains as well as alter cellular responses such as growth factor binding and activation, cell attachment, and angiogenesis (Schuksz, 2008). The expected result to GAG binding would be less CS56 expression since GAG surfen would hypothetically decrease GAG production.

Another idea for the future would be to treat cells with radiation or chemotherapy after introducing F98 cells in order to see how these treatments would affect the growth, migration, and spread of the GBM tumor. By investigating cell migration and using immunohistochemistry in addition to a treatment method, we would be able to investigate glioma tumors in a context more like that of an actual GBM patient. If a sulfation-driven GAG mechanism were to be discovered, a subsequent potential source of investigation would be to use CRISPR to edit the genome of the glioma tumor to silence the sulfation mechanism which would essentially stop growth of the glioma tumor, assuming that there is a sulfation component in proliferation.

In summary, the slice culture model successfully bridges the gap between a need for an *in vivo* and *in vitro* model.

VII. References

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