Hydrostatic Pressure Induces Glioblastoma Cell Death in Tumor Edema Model

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Introduction

Glioblastoma is a primary brain tumor typically characterized radiologically by a contrast-enhancing lesion and a midline shift. Tumor growth, even in the presence of chemoradiation, eventually compromises neurological function, yielding a median patient survival time of approximately 14 months. Glioblastoma cells are often found migrating through the brain parenchyma along small blood vessels [1, 2]. Specifically, they migrate between the astrocyte end processes and the outer layer of the blood vessel. This disrupts the natural blood-brain barrier (BBB) [2] (Fig. 1b). In addition, secretion of VEGF-A causes the sprouting of new, leaky, inefficient blood vessels [3]. Further, there is a lack of drainage as the brain does not have a lymphatic system and new veins are not developed during angiogenesis. In turn, the interstitial fluid pressure (IFP) in the tumor rises close to microvascular pressure levels [4].

Elevated IFP has mainly been described by Rakesh Jain’s group where it has been attributed to blood stasis, hypoxia, and a decrease in drug delivery to the tumor [5]. While the Jain group has mainly focused on vessel normalization and the effective decrease in IFP [6], there are few studies on the effect of IFP directly on tumor cells [7-9], and to our best knowledge, none before now on the effect of IFP on glioblastoma cells.

Physical forces in the tumor microenvironment represent an innovative opportunity to find new drug targets for treating tumors, especially highly lethal tumors like glioblastoma. By understanding the effect of the physical force on the molecular level, we can introduce inhibitors
to modify the phenotype [10]. Here, we measure glioblastoma cell viability, mechanism of cell death, and molecular signaling in the context of physiologically relevant levels of IFP.

2. Materials and Methods

2.1 Cell Culture

Each of the U87, U251, and LN229 cell types were cultured according to standard cell culture techniques. Briefly, cells were cultured in DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum (VWR), 1% penicillin/streptomycin (VWR), and 0.1% Mycozap (VWR), and passaged at ~80% confluency.

2.2 Size

Cells were trypsinized and suspended cells were analyzed using a Cellometer Auto T4 Cell Counter (Nexcelom Bioscience). The diameter of each cell was recorded and averaged. The average diameter was converted to surface area, assuming a spherical shape, which was observed during experimentation.

2.3 Migration

Cells were stained with CellTracker Green (ThermoFisher Scientific) and seeded at ~20k per well in a 12 well plate. After 18 hr, the media is replaced to remove any non-adherent cells, and the plate is placed in a live-cell imaging chamber maintained at 37°C and 5% humidity. Time-lapse microscopy is performed over an 18 hr period, videos are produced, and single cell migration speed is measured using ImageJ.

2.4 Proliferation
Cells are seeded on a 12 mm diameter coverslip in a 12 well plate at ~40k per well and allowed to adhere for 18 hr. Cells are then pulsed with EdU at 10 μm for 2 hr. Cells were then immediately fixed with 3.7% paraformaldehyde and permeated with 0.5% Triton X-100. EdU was labeled with Alexa Fluor 488 (AF488) and nuclei were labeled with Hoechst 33342. Fluorescence images were taken in random positions, and the ratio of AF488 positive cells to Hoechst 33342 positive cells was calculated to determine proliferation rate.

2.5 Pressure Selection

In order to examine IFP in an in vitro setting, it is useful to first summarize the range of physiological pressures in the brain with and without tumor. Physiological pressure in the brain varies greatly and is difficult to assess because intracranial pressure (ICP) cannot be measured in tumor patients without risking herniation and death. Further, ICP does not necessarily approximate IFP with a tumor present. However, some reasonable estimates for IFP can be obtained. When lying down, cerebrospinal fluid pressure (CSFP) is 8-15 mmHg and approximates ICP, which can be assumed to be approximately equal to IFP in normal, healthy adults (Intracranial Hypertension Research Foundation). Patients with brain tumors have been reported to present with mean CSFP between 24-33 mmHg [11-13]. In the presence of brain lesions, IFP is similar to or higher than CSFP [14]. Neurological deficits are often evidenced with intracranial hypertension (i.e., ICP ≥ ~20mmHg) (Intracranial Hypertension Research Foundation). Furthermore, as microvasculature is the driving force for local increases in IFP, the maximum IFP is likely close to microvascular pressure levels. The best estimate of microvascular pressure can be taken from evaluating the relatively small pial arteries, and has been determined to be ~60 mmHg [15]. Taken together, these data suggest that IFP in healthy individuals is 8-15 mmHg and is likely 20-55 mmHg in tumor patients.
2.6 Pressure Application

Hydrostatic pressure was controlled using a pressure apparatus developed in our lab (Figure 1). Sterilization is performed by ethanol perfusion followed by perfusion with phosphate buffered solution, and then air to dry. For each experiment, cells of varying quantity were seeded in a 12 well plate and allowed to adhere for 18 hr. Then, the media in each well is replaced after one gentle rinse with full media to remove any non-adherent cells. The rubber stoppers of the pressure apparatus are inserted in to each well, and then the clamp is screwed to the well plate. The pressure level is set to the desired level, and monitored and adjusted as necessary over the 6 hr exposure time.

Figure 1. Method of modulating fluid pressure during cell experiments. Schematic (A) and photograph (B) of the pressure apparatus.

2.7 Viability Assay

Cells were seeded at ~80k per well in a 12 well plate and allowed to adhere for 18 hr. Each cell type is then subjected to each pressure level for 6 hr. At the end of the pressure exposure, viability was measured using flow cytometry. Live cells were positive for C12-Resazurin and dead cells
were positive for SYTOX Green. Positive and negative controls were used, where dead cells were induced by incubating at 55°C for 60 min.

**2.8 Apoptosis/Necrosis Assay**

Cell death studies were similar to viability experiments, where cells were seeded at 80k per well in a 12 well and allowed to adhere for 18 hr. Following a 6 hr pressure treatment, apoptosis/necrosis was measured using flow cytometry. Apoptotic cells were either positive for only annexin v, or for both annexin v and propidium iodide (PI) indicating either early or late apoptosis, respectively. Classification of the two was not noted. Necrotic cells were positive for only PI, and live cells were negative for both stains.

**2.9 Western Blot**

Cells were seeded at 320k per well in a 12 well plate for 18 hr. After 6 hr at 22.4 mmHg, cells were gently rinsed, then lysed and processed for western blotting. Blots were labeled for Beta-1 integrin (Abcam), phospho-Akt (Cell Signal), and Actin (Sigma) as a loading control.

**2.10 Statistical Analysis**

Statistical significance at alpha level 0.05 was determined using ANOVA with post ad hoc Tukey’s tests, or a Student’s T Test, where appropriate.

**3. Results**

**3.1 Cell Characterization**

A hallmark feature of glioblastoma tumors is genetic heterogeneity [16]. This leads to difficulty in treatment, as well as potential difficulty in describing tumor cell phenotype *in vitro* with
established cell lines. To account for this variability, we employ a panel of cell lines and characterize their surface area, migration speed, proliferation rate (Figure 2). Hydrostatic pressure is related to surface area such that at a given hydrostatic pressure, an object with a larger surface area will experience a greater net force. The U87 cell line has a surface area statistically significantly larger than either the U251 or LN229 cell lines by a factor of ~1.3. The U87 cells also feature the largest migration speed, while the U251 cells are the slowest migrating. Lastly, the LN229 cells proliferate much quicker than either the U87 or U251 cell lines by a factor of >1.5.

![Cell characterization](image)

Fig 2. Cell characterization. Cells were characterized for surface area (A), migration speed (B), and proliferation rate (C). Data is mean ± standard error, and levels with a star are statistically significant as compared to one another.

### 3.2 Cell Survival

The ideal glioblastoma treatment will prioritize tumor cell death as opposed to secondary considerations, such as migration speed or proliferation. As such, we investigate the effect of hydrostatic pressure, at physiological levels, on glioblastoma cell death (Figure 3). We apply different levels of fluid pressure and observe a parabolic curve in viability with the minimum at the level of IFP observed in a healthy adult. Tumor cell viability then rises closer to the level observed in the atmospheric control as the fluid pressure rises closer to microvascular pressure.
This phenomenon is observed across all three cell types, however the U251 and LN229 cells are less sensitive to the fluid pressure, with a minimum viability ~1.5x higher than the U87 cells.

![Graph showing cell survival at 6hr.](image)

Fig 3. Cell survival at 6hr. Data is mean ± standard error, and levels connected by a star are statistically significant as compared to one another.

**3.3 Apoptosis/Necrosis Assay**

The scope of this parcel of work is to make observations that are potentially clinically relevant. As such, while there are a number of potential follow up experiments based on the previously undescribed parabolic viability response to hydrostatic pressure, we necessarily limit ourselves here to further probing the mechanism of cell death (Figure 4). We apply our assumed ‘Low Tumor’ fluid pressure of 22 mmHg and find that the clear majority of cells (i.e. >95%) are apoptotic across all three cell types.
Fig 4. Cell death. Dead cells are classified as either apoptotic or necrotic. Data is mean ± standard error, and levels connected by a star are statistically significant as compared to one another.

### 3.4 Apoptosis Survival Pathway

The down regulation of apoptosis, or programmed cell death, is a core pillar of tumorigenesis [17]. As a result, we are interested in how hydrostatic pressure induces significant apoptosis in glioma cells. Specifically, we probe what molecular signaling pathways are altered in response to hydrostatic pressure. We look at p-Akt, a molecule that helps tumor cells evade apoptosis [18], and beta-1 integrin, an over expressed membrane-bound adhesion molecule in glioma [19], at our ‘Low Tumor’ fluid pressure of 22 mmHg (Figure 5). We find that in U87 and U251 cells, p-Akt
expression is lower under pressure, while it is higher in LN229 cells. Across all three cell types, beta-1 expression is reduced to a negligible level under fluid pressure.

![Western blot of glioblastoma cells at atmospheric pressure and 22 mmHg.](image)

**Discussion**

Glioblastoma cells are killed under hydrostatic pressure, which is representative of IFP *in vivo*. Interestingly, the lowest level of viability across all 3 cell types is at 9 mmHg, which is within the range of healthy adult IFP. Further, this minimum is ~1.5x higher in the U251 and LN229 cells than in the U87 cells, while the U87 cells are ~1.5x larger in surface area. This correlation may be the result of a higher net force experienced by the U87 cells, which leads to a stronger response mechanism eventually leading to more cell death than in the smaller U251 and LN299 cells. This result, along with the parabolic viability response to fluid pressure will be the subject of future studies.
The finding that cell death is primarily apoptotic is followed by a probe of the signaling pathways involved. Expression of p-Akt is decreased in the presence of pressure in the U87 and U251 cell lines, but is increased in response to pressure in the LN229 cells. Protein expression is measured in the remaining adherent cells after pressure exposure. So, it is possible that the decreased p-Akt is part of the mechanistic response to fluid pressure in many cells. Without p-Akt, an apoptosis inhibitor, cells are more susceptible to apoptosis cues and die. The LN229 cell increase in p-Akt might be the result of adaptation of the local cell population within the well plate. Given the genetic heterogeneity of glioblastoma cells and the advanced proliferation rate of LN229 cells as compared to the other cells, it is possible that the elevated IFP acts as a selective pressure. Within this scenario, the result would be a population of cells that are more fit to survive the selective pressure (i.e. increased p-Akt expression under elevated fluid pressure). This hypothesis will be studied further in future experiments. In addition, expression of beta-1, an integrin overexpressed in glioma and responsible for cell adhesion, is effectively negated under pressure. This may be related to the sensing of the elevated fluid pressure, or to a cell response. Further experiments are warranted to determine how cells sense and respond to elevated fluid pressure on a molecular signaling level.

While our results have been rigorously obtained, we offer that there are some limitations. Measuring the ICP in the brain via lumbar puncture while a tumor is present results in herniation. This limits our ability to quantify IFP in humans. ICP in rodents and other smaller mammals is much lower, ruling out the possibility of using a surrogate animal [14]. Because we know that microvascular pressure is the driving force for the elevated fluid pressure in tumors [20], we can take advantage of approximate values to set our range in this study. Further, the most interesting
finding is at 9 mmHg, which is well within accepted measures of IFP in the brain and gives strength to our conclusions.

References