Glioblastoma is the most prevalent and one of the most malignant brain tumors, with a dismal median survival rate of approximately 1 year. Current standard of care therapy: surgical resection, adjuvant radiation, and Temozolomide (TMZ), can prolong the survival of patients by a couple of months, but despite these treatments the 5-year survival rate continues to be <2%. This standard of care was established in 2005 and has not changed since. Recent advances in cancer immunology, though, have revealed immunotherapies to be a promising approach for many types of cancer, and this paradigm is being actively explored in glioblastoma.

The immune system can be exploited on multiple different levels to achieve an anti-tumor immune response: cytokine therapies, vaccines, and immune checkpoint modulators have all been explored. Immune checkpoint modulators are some of the most promising of these approaches, with multiple therapies being FDA approved for certain cancers. As seen in figure 1, surface molecules such as PD-L1 expressed by tumor cells act as co-inhibitory molecules, downregulating the anti-tumor immune response. Immune checkpoint modulators use antibodies to block this interaction, allowing for a more robust anti-tumor response.

The Fas-FasL interaction between T-cells and tumor cells is more complicated. Depending on the expression of certain genes like perforin and c-FLIP, expression of Fas on tumors can help the T-cells destroy the tumor or help the tumor cells destroy the T-cell.

It is likely that the future of immunotherapy lies in its synergism with current frontline cytotoxic therapy. Thus, it is important to understand how these two therapies interact with one another. In glioblastoma that starts with understanding how TMZ and radiation affect the expression of these immune markers.

Figure 1. Tumor cells use immune checkpoint molecules to modulate the immune response.
Differential Effects of Chemotherapy and Radiation on Immunoregulatory Markers in Primary versus Recurrent Glioblastoma

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MATERIALS & METHODS

**Cells:** GBM10 & GBM43 cells were acquired from the Mayo Clinic. GBM10 is a recurrent glioblastoma cell line, while GBM43 is a primary glioblastoma cell line. Cells were cultured in DMEM supplemented with 10% FBS and 1% HEPES, and maintained in an incubator at 37° with 5% CO2.

**Treatment:** Cells were seeded in 6-well plates overnight, then treated 24 hours later with 200mM of TMZ. Cells that were subject to 3000 cG of radiation were done so immediately before initially seeding the 6-well plates. Cells were then incubated for 72 hours before collection, and expression of cell surface markers was evaluated via Flow Cytometry using anti-PD-L1-PE and anti-Fas-FITC antibodies.

**Tumorspheres:** Tumorspheres were formed by seeding a flat-bottom, ultra-low attachment surface 6-well plate with 150,000 cells, and incubating them with neural stem cell media under hypoxic conditions. Cells that were subject to 3000 cG of radiation were done so immediately before initially seeding the plates. After a 24-hour incubation the cells were treated with 200mM of TMZ. The cells were then incubated for 72 hours, and their picture was taken. This was performed by Dr. Reza Saadatzadeh.
RESULTS

GBM10, a recurrent Glioblastoma cell line, expresses higher levels of both PD-L1 and Fas than the primary GBM43 cell line

The GBM10 and GBM43 cell lines were acquired from the Mayo Clinic in Rochester, MN. The GBM43 cell line was established from a primary tumor that had not received any prior treatment, while the GBM10 cell line was established from recurrent glioblastoma that had previously been exposed to radiation and TMZ.

Whole genome sequencing on these cells, performed by the New York Genome Center, revealed mutations in several important genes in each cell line. GBM43 cells were reported to have mutations in TP53 (Phe270Cys) and NF1 (Arg416* stop) genes, while GBM10 cells were reported to have a mutation in PTEN (Ile135Val).

![Figure 2](image_url)

Figure 2. Expression of both Fas and PD-L1 in GBM10 and GBM43 cell lines. Cells were seeded in 6 well plates, incubated at 37°C for 72 hours, collected, then analyzed for Fas and PD-L1 expression using flow cytometry. GBM10 cells were found to have significantly higher PD-L1 expression than GBM43 cells, and while the Fas levels in GBM10 cells were higher than GBM43 the results were not significant.
RESULTS

TMZ induces both Fas and PD-L1 expression in primary glioblastoma cells, but not in recurrent glioblastoma cells

Figure 3. The effect of TMZ treatment on PD-L1 and Fas expression in GBM10 and GBM43 cells. Cells were seeded in 6-well plates for 24 hours, then were treated with 200mM of TMZ. 72 hours later these cells were collected and analyzed for expression of PD-L1 and Fas using flow cytometry. (A) PD-L1 expression is induced in GBM43 cells treated with TMZ. (B) PD-L1 expression in GBM10 cells remains the same after treatment with TMZ. (C) Fas expression in GBM43 cells is induced after treatment with TMZ. (D) Fas expression in GBM10 cells remains the same after treatment with TMZ.
RESULTS

Radiation induces PD-L1 expression in primary glioblastoma cells, but not recurrent glioblastoma cells

Figure 4. The effect of radiation on the proportion of PD-L1 expressing cells in GBM10 and GBM43 cells. Cells were collected from flasks, then subject to 3000cG of radiation for 5 minutes. They were then plated in 6-well plates, and incubated for 72 hours before collection and analysis via flow cytometry was performed. Significantly more GBM43 cells were found expressing PD-L1 after exposure to radiation, compared to cells that were not exposed to radiation. This is in contrast to the GBM10 cells, where radiation did not significantly change the proportion of PD-L1 expressing cells.
RESULTS

Using tumorspheres to better model the tumor microenvironment

Figure 5. Comparison of adherent cell cultures to tumorspheres. GBM10 cells were expanded in standard tissue culture (adherent cells) or neural stem cell media for 24 hrs, 48 hrs, and 3 weeks.

Figure 6. Visualizing GBM10 and GBM43 tumorspheres before and after treatment with TMZ (T) and radiation (R). (A) GBM10 and (B) GBM43 cells were either irradiated or not, expanded in neural stem cell media for 24 hours, and then either treated with 200mM TMZ or not. Pictures were taken 72 hours after treatment.
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Discussion

We were able to show that cells derived from recurrent glioblastoma tumors (GBM10) expressed higher levels of PD-L1 compared to cells derived from primary glioblastoma tumors (GBM43). We hypothesized that this result may be due to those cells’ previous exposure to radiation and TMZ. We were then able to show that both radiation and TMZ play a role in regulating the expression of PD-L1 in the GBM43 cells. The reason for the increased expression of PD-L1 on GBM10 cells may also be due to the mutation these cells have in the PTEN gene. It has previously been reported that loss of the tumor suppressor PTEN increases the expression of PD-L1 through an overactive PI(3)K/Akt pathway.

While the differences in Fas expression in GBM10 versus GBM43 cells were not significant, we were able to show that GBM43 cells treated with radiation and TMZ experience a significant upregulation of Fas expression. GBM10 cells treated with radiation or TMZ did not experience this upregulation. It has been previously shown that human glioma cells express high levels of Fas, but the sensitivity of these cells to Fas-mediated death remains variable. Fas is able to be used by the immune system to kill tumor cells, but it can also play an angiogenic and pro-inflammatory role in glioblastoma. Even further, the Fas-FasL interaction between tumor cells and cytotoxic T-lymphocytes (CTL) may lead to the killing of CTLs infiltrating the tumor. The specific role this Fas expression plays in the tumor microenvironment is an area we hope to explore further.

REFERENCES


We are currently using tumorspheres of GBM10 and GBM43 cells to investigate the expression of PD-L1 and Fas using western blot. This cell culture technique has been shown to better recapitulate the tumor microenvironment than adherent cell cultures. We hope to use it to gain a better understanding of how treatment may affect the expression of these immunoregulatory molecules.
Summary Points

- Often patients who are enrolled in clinical trials have been exposed to standard therapy previously, in the case of Glioblastoma this is Temozolomide (TMZ) and radiation.

- We investigated how this standard therapy may change the immune markers on cancer cells, and thus may affect the response patients have to immunotherapies.

- Tumor cells derived from recurrent glioblastoma tumors (GBM10) expressed higher levels of PD-L1 at baseline compared to cells derived from primary tumors.

- Both radiation and TMZ upregulated the expression of PD-L1 in the primary tumor cell line.

- While differences in Fas expression in GBM10 versus GBM43 cells were not significant, we were able to show GBM43 cells treated with TMZ experience a significant upregulation of Fas expression.

- Tumorspheres were also created to potentially better model the tumor microenvironment in future experiments.