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An experimental xenograft mouse model of diffuse pontine glioma designed for therapeutic testing

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Abstract

The prognosis for diffuse infiltrating pontine gliomas (DIPG) remains extremely poor, with the majority of patients surviving less than 2 years. Here, we have adapted standard xenograft techniques to study glioma growth in the mouse brainstem, and have utilized the mouse model for studying a relevant therapeutic for treating DIPGs. bioluminescence imaging monitoring revealed a progressive increase in signal following the injection of either of two tumor cell types into the brainstem. Mice with orthotopic GS2 tumors, and receiving a single 100 mg/kg dose of temozolomide showed a lengthy period of decreased tumor luminescence, with substantially increased survival relative to untreated mice (P<0.001). A small molecule inhibitor that targets cdk4/6 was used to test AM-38 brainstem xenograft response to treatment. Drug treatment resulted in delayed tumor growth, and significantly extended survival. Our results demonstrate the feasibility of using an orthotopic brainstem tumor model in athymic mice, and for application to testing therapeutic agents in treating DIPG.

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Conflict of interest None.

Keywords

Brainstem glioma; Mouse model; Bioluminescence image

Introduction

Brainstem tumors arise predominantly in the pediatric population where they constitute up to 20% of childhood brain tumors [1, 2]. The majority of brainstem tumors are diffuse infiltrating pontine gliomas (DIPG), and patients with these tumors rarely survive beyond 2 years from initial diagnosis [3]. Although many chemotherapy and radiation therapy regimens have been used to treat DIPGs, none has proved successful in conferring a survival advantage, compared to radiation alone [4]. Factors which contribute to the dismal prognosis of patients with DIPG include their anatomic location which prevents meaningful surgical resection, and their invasive dissemination [5].

Progress has been made in the treatment of high grade supratentorial glioma using targeted anticancer agents [6–10]. The applicability of anatomically accurate model systems to test the efficacy of therapeutic agents has also been demonstrated [2]. We recently reported the development of an orthotopic brainstem tumor model in athymic rats using GS2 glioma cells [11]. However, there are limitations of the rat model, primarily associated with the high expense of housing, imaging cost, and acquisition of sufficient drug for testing therapy-response hypotheses.

Using human GS2 and AM-38 human glioma cells, we report the development of an orthotopic brainstem tumor model in athymic mice. In addition to determining the technical parameters required for implantation of tumor cells into the mouse brainstem, we also tested the applicability of this model system as platform for therapeutic testing of new agents. We examined the effect of a relatively non-specific agent such as temozolomide (TMZ), as well as the small molecule inhibitor of CDK 4/6, PD-0332991, with the latter being effective against human DIPG cell growth in vitro.

Materials and methods

Cell culture

GS2 human glioma cells were obtained from Manfred Westphal (Hamburg, Germany), and maintained as a neurosphere culture in Dulbecco's modified Eagle's medium High Glucose (DME H-12), 100 U/ml penicillin (Invitrogen), B-27 supplement (50×) without vitamin A (Invitrogen), N-2 supplement (100×) (Invitrogen), 20 μg/ml EGF (Calbiochem) and 20 ng/ml FGF (Sigma, St Louis, MO, USA).

AM-38 human glioma cell were obtained from Japan Health Sciences Foundation Health Science Research Resources Bank [12]. AM-38 cells were grown as adherent monolayer cultures in Dulbecco's modified Eagle's medium High Glucose (DME H-21), without Phenol Red (UCSF Cell Culture Facility), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

Cells were transduced with a lentiviral vector containing firefly luciferase (FL) under the control of the spleen focus forming virus (SFFV) promoter. Lentiviral vectors were generated by transfection of 293T cells with plasmids encoding the vesicular stomatitis virus G envelope, gagpol, and Fluc genes. Conditioned medium containing viral vectors were harvested 48 h post transfection, filtered (0.45 mm), and frozen until use. Cells were screened for transfection efficiency by treatment with luciferine (D-luciferin potassium salt,

150 mg/kg, Gold Biotechnology, St Louis, MO) in vitro and examination by a Xenogen IVIS Lumina System (Xenogen Corp., Alameda, CA). More than 80% of cells were transfected [11].

Animals

Five-week-old female athymic mice were (Simonsen Laboratory, Gilroy, CA) maintained in a temperature and light controlled animal facility under pathogen-free conditions in air-filtered cages and received food and water. Mouse weights were recorded daily. If an animal's weight dropped 15% below baseline, treatment was withheld until the weight recovered. Animals were sacrificed if they became symptomatic from brainstem tumor burden according to the protocol. All animal protocols were approved by the UCSF Institutional Animal Care and Use Committee.

Surgical technique

Localization of tumor cell implantation in the pons was determined by initially using blue dye in cadaveric mice. Prior to surgery mice were anesthetized using an intraperitoneal injection of 100 mg/kg of ketamine and 10 mg/kg of xylazine. The skull of the mouse was exposed and a small opening was made using 25 gauge needle (PrecisionGude) 1.5 mm to the right of midline, posterior to the lambdoid suture. GS2 FL or Am38 FL cells were injected into the pons (5.0 mm depth from the inner table of the skull) with a 26 gauge Hamilton syringe (Hamilton Company, Nevada, USA). A total of 1.0×10^6 GS2 FL cells or 1.0×10^4 Am38 FL cells in a volume of 0.5 μL were injected using a free hand technique [13].

Therapy response experiments

Athymic mice implanted with luciferase-modified GS2 or AM-38 glioblastoma cells were randomized to vehicle control (OraPlus, for TMZ, 50 mM sodium lactate, pH 4, for PD-0332991) or TMZ treatment (Temodar, Schering-Plough, Kenilworth, NJ) or PD treatment (PD-0332991, Pfizer Inc, NY) groups. For GS2 glioma xenografts, the treatment group ($n = 5$) received a daily dose of 100 mg/kg of TMZ by oral gavage on day 30. For AM-38 glioblastoma xenografts, the treatment group received a daily dose of 150 mg/kg PD-0332991 by oral gavage for 12 consecutive days. All mice were monitored every day for the development of symptoms related to tumor burden and twice weekly by bioluminescence imaging (BLI) (see below). Mice were euthanized when they exhibited symptoms indicative of significant compromise to neurological function. Animal survival was defined as the time euthanasia was performed.

In vivo bioluminescence imaging

In vivo BLI was performed with the Xenogen IVIS Lumina System (Xenogen Corp., Alameda, California) coupled to the data-acquisition Living-Image software (Xenogen Corp., Alameda, California) [14, 15]. Mice were anesthetized with 100 mg/kg of ketamine and 10 mg/kg of xylazine and BLI was performed 10 min after intraperitoneal injection of 100 μl D-Luciferin (150 mg/kg) (Gold Biotechnology, St. Louis, Missouri). All mice were imaged 1 or 2 times per week. Signal intensity was quantified within a region of interest over the heads of mice, as defined by the Living-Image software. To facilitate comparison of growth rates, each mouse's luminescence readings were normalized against its own luminescence reading at the day of last BLI before initiation of therapy, thereby allowing each mouse to serve as its own control. Previously published data using these techniques documented a strong correlation between measured bioluminescence, tumor burden, and animal survival [11].

Immunohistochemistry

All rodent tissues were fixed in phosphate-buffered 10% formalin dehydrated by graded ethanol, and then embedded in wax (Paraplast Plus, McCormick Scientific, St. Louis, Missouri). Sections 5 μm thick were cut from embedded mouse brainstem samples. Hematoxylin and eosin (H&E) staining was performed according to standard procedures. For immunohistochemistry, Mib-1 labeling and Caspase-3 stained with primary antibody by UCSF IHC and molecular pathology core facility. After relative quantification of these staining, representative slides were recorded. Positive cells were determined by EVOS microscope (AMG (Advanced Microscopy Group), WA, USA) and the numbers of positive cells were countered in four high-power fields (40× magnification) from each mouse, and mean values determined.

Statistical analyses

The Kaplan–Meier estimator was used to generate the survival curves [16] and differences between survival curves were calculated using a log-rank test. Differences between the two groups with Mib-1 and Caspase 3 positive cells were compared using a student's *t* test [17] using Prism software (GraphPad Software, San Diego, CA, USA).

Results

Characterization of the GS2 xenograft model

There are technical challenges associated with implantation of tumor cells into the rodent brainstem. The brainstem itself is compact in size and rapid, procedure-associated development of symptoms, such as torticollis and nystagmus, necessitate animal euthanasia. In our model system, BLI monitoring revealed successful tumor cell engraftment in all animals, and progressive tumor growth in each case. Implanted tumors demonstrated logarithmic growth subsequent to day 7 after implantation, at which time injected mice were asymptomatic. Animals were euthanized once symptoms described above developed. The median survival, as defined by the onset of symptoms, was 54 days after implantation, with a range of 50–62 days.

In a time course study used to evaluate accuracy of monitoring tumor growth by BLI, mice were euthanized at pre-determined time points, and immediately following BLI (Fig. 1a), with whole brains removed, processed, and cut to obtain serial cross sections in the axial plane. Luminescence signals detected in right pons on day 20, 30 and 40 indicated progressive tumor growth (Fig. 1a), with GS2 tumor easily visible in brain by day 30 post tumor cell injection, and showing an increasing degree of brain infiltration after day 40. On day 50, the implanted tumor was seen within the right pons and in some cases, with extension over the surface of the brainstem (Fig. 1b).

Tumor response to temozolomide treatment

We next measured GS2 brainstem xenograft response to TMZ by BLI monitoring. Mice were randomized into vehicle only and TMZ treatment groups on day 30 after tumor implantation, with the TMZ group receiving a single 100 mg/kg dose of TMZ on day 31. The normalized luminescence plot for the control group showed progressively increasing luminescence until mice developed neurological symptoms indicative of a moribund condition. In comparison to the control group, the luminescence signal in mice receiving TMZ treatment decreased and remained low until day 80 following implantation (Fig. 2a–c). The median survival of mice treated with TMZ was significantly extended relative to controls ($P = 0.0006$; Fig. 2b).

The effect of therapy on tumor cell proliferation was additionally assessed using MIB-1 staining. As expected, there was a significant reduction in MIB-1 positive staining in the TMZ-treated mice as compared to the control group (** $P<0.001$, Fig. 3a). In mice that were sacrificed after TMZ treatment, caspase 3 staining was used to evaluate the effect of treatment on GS2 tumor cell apoptosis. The number of caspase 3 positive cells were significantly increased in mice treated with TMZ (** $P<0.001$, Fig. 3b).

Tumor Response to PD-0332991 Treatment

A primary cell line derived from a patient with a DIPG that we have characterized for genomic alterations (manuscript in preparation), was determined to have homozygous deletion of CDKN2A, which encodes p16 (Fig. 4). In addition, we have shown that a cdk4/6 inhibitor, PD0332991, is effective in slowing the growth of supratentorial malignant xenografts harboring activated cdk4/6 by virtue of homozygous inactivation of p16. The glioblastoma cell line AM-38, that also lacks p16, showed significantly reduced proliferation in vitro when treated with PD0332991 (unpublished data). AM-38 was used to establish brainstem tumors in athymic mice to test the in vivo efficacy of PD0332991 in association with DIPG anatomic modeling. Mice receiving PD0332991 treatment experienced reduced tumor growth and extended survival relative to control mice (Fig. 5).

Discussion

In this study, we describe the development of a mouse model for brainstem human glioma that has been developed to facilitate the pre-clinical testing of new therapeutic agents for treating DIPG. Previously, we demonstrated the feasibility of implanting and monitoring an brainstem glioma xenografts in rats [11]. However, the rat model has limitations, primarily associated with expense, including purchase, housing, imaging, and acquiring sufficient drug to conduct therapeutic tests.

Very few studies have used an orthotopic xenograft model for examining brainstem glioma growth. The mouse pons is very small, and inaccurate targeting of implanted cells results in extension of tumor growth outside the brainstem. Furthermore, inaccurate tumor cell injection may lead to increased procedural mortality. Here, we used two established human cell lines, GS2 and AM-38, to establish brainstem xenografts in mice. We had previously observed that each cell line grows invasively when propagated in the supratentorial compartment, and our results demonstrate their progressive, infiltrative growth in the mouse pons. A limitation of our experimental system is that both GS2 and AM-38 are derived from adult high grade astrocytomas, and not from pediatric DIPGs. In the current study we utilized a DIPG cell line (SF7761) for testing therapeutic response in vitro. Unfortunately this cell source is only weakly tumorigenic, and produces very slow growing tumors in athymic mice that are not compatible with therapeutic testing. Consequently we used the two GBM cell lines for in vivo anatomic modeling and investigating brainstem tumor response to therapy.

TMZ is commonly used to treat supratentorial high grade tumors in a clinical setting but is not generally effective for patients with DIPG. However, TMZ is a useful tool to study the response of intracranial tumors since most cell lines show some sensitivity to this drug. TMZ reduced the luminescence of GS2 brainstem tumors, and increased animal survival. The use of BLI allows quantitative measurement of tumor growth, which correlates well with tumor volume. From a practical perspective, the per animal cost of mouse bioluminescence imaging is approximately tenfold less than for rats.

We also chose to study an enzymatically targeted small molecule inhibitor in our model system. It has been reported that the activation of cyclin-dependent kinases 4/6 (cdk4/6) is

among the most common aberrations found in human cancer [18], and occurs in the majority of glioblastoma [19–21].Arecent report showed that a CDK4/6 inhibitor was effective in supratentorial GBM rodent model [12]. We examined whether PD0332991 (an inhibitor of CDK4/6) would extend survival in mice with brainstem xenografts by reducing tumor growth rate. For these studies, we used glioblastoma cell line AM-38 because it has homozygous deletion of p16, as is the case for the DIPG cell line we have established and tested for in vitro sensitivty to PD0332991. BLI and survival of mice with AM-38 brainstem tumors indicated that there was antitumor activity of PD0332991.

In summary, our results, along with those from other groups, demonstrate the feasibility of using an orthotopic brainstem model for pre-clinical therapeutic testing in mice [22–26]. The development of a panel of tumorigenic cell sources from DIPG patients, for use in a brainstem xenograft context, will further advance this approach for identifying effective therapeutics for treating pediatric patients afflicted with this cancer.

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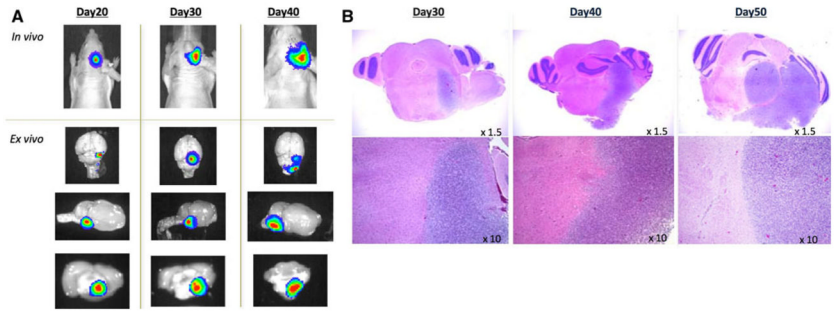

Fig. 1.

Time course study monitoring tumor growth by BLI and histopathology analysis. **a** Mice received brainstem injection of 1×10^6 luciferase-modified GS2 cells. Increasing luminescence signals were detected, consistent with progressive tumor growth in the pons. **b** In a time course study, mice were euthanized on day 30, 40 and 50 and GS2 tumor growth was assessed by histopathology analysis. GS2 tumor detected within right pons on day 30 after implantation and showed increasing degree of infiltration after day 40. On the day 50, tumor was evident in the right pons with extension over the pons surface. Infiltrating tumor in the medulla is seen with pons tissue

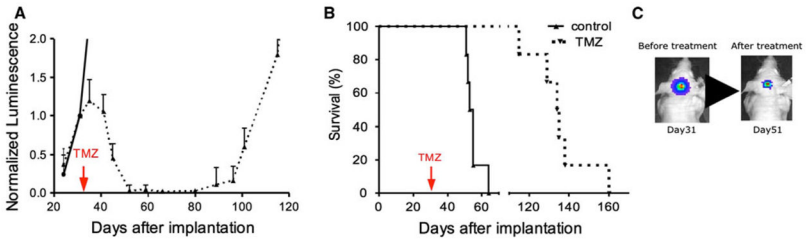

Fig. 2.

Mice were randomized to control and Temozolomide (TMZ) treatment groups. **a** BLI showed decreasing normalized luminescence in the TMZ treatment group. **b** Survival graph showing the TMZ treatment group significantly prolonged median survival ($P = 0.0006$) from day 54 (range of 50–62 days) in the control group to 134.5 days (range of 110–160 days) in the TMZ treatment group. **c** Survival correlated with luminescence readings

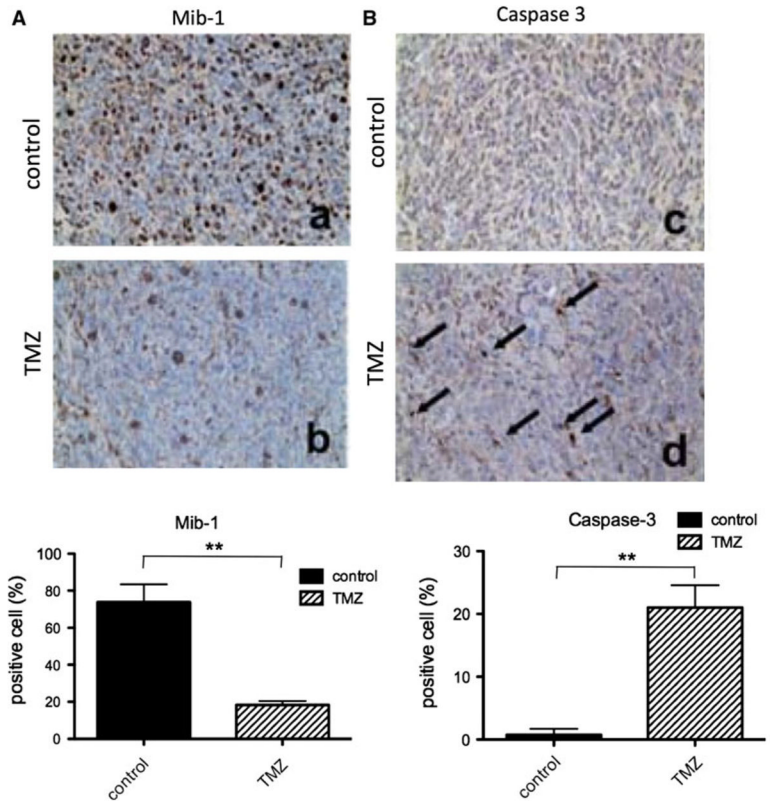

Fig. 3.

The effect of TMZ therapy on proliferation and apoptosis. **a** Increased numbers of Mib-1 positive cells were present in control animals as compared to those animals receiving 1 week of TMZ treatment. The number of Mib-1 positive cells were significantly reduced in the treated animals compared to controls (mean value = 73.55 control vs. 18.25 TMZ treated). **b** Active caspase-3 is not seen in the control animals. Caspase 3-positive cells were significantly present in the GS2 tumors following TMZ treatment compare to control (mean value = 0.75 control vs. 42.75 TMZ treated)

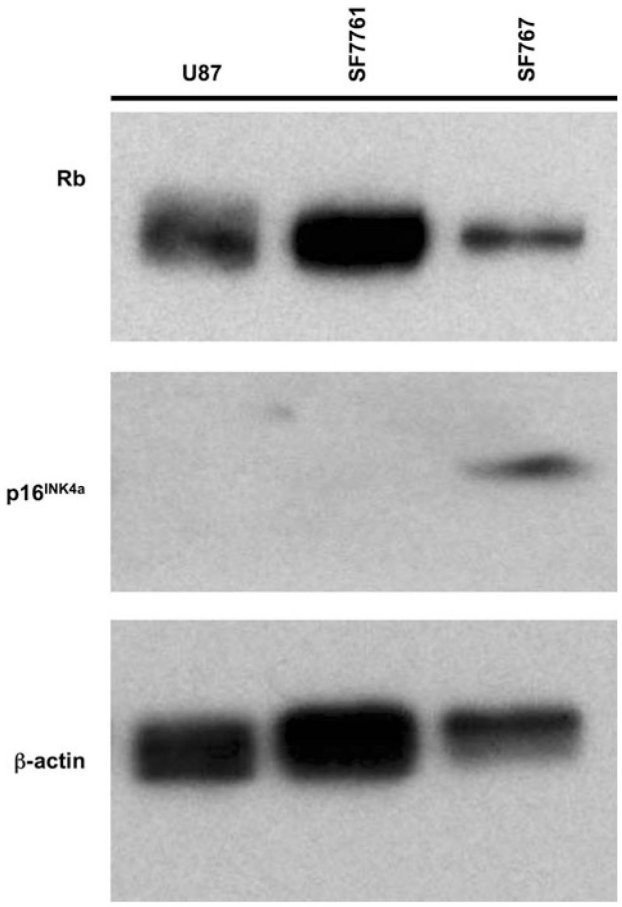

Fig. 4.

Protein expression of Rb and p16. Protein expression for three glioma cell lines were examined for Rb and p16 protein expression. U87 (malignant glioma) and SF7761 (pediatric DIPG) express Rb but lack p16 expression, while SF767 (malignant glioma) has a reduction in Rb with p16 present

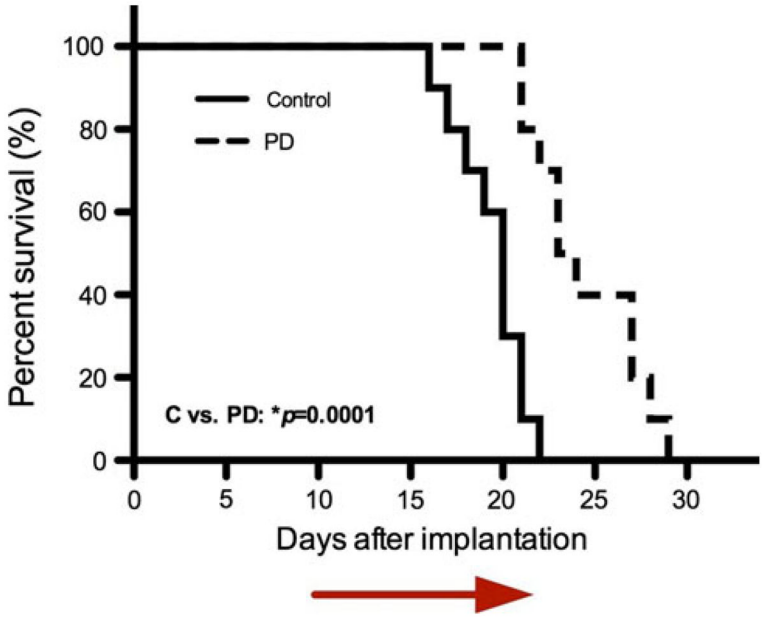

Fig. 5.

Treatment with PD-0332991. Mice were randomized to two groups (control, PD-0332991). The survival analysis showed prolonged survival in the treatment group (control vs. PD: $P = 0.0053$), from day 19 (range of 16–22 days) in the control group to 24 days (range of 22–29 days) in the TMZ treatment group