Establishment of a human glioblastoma stemlike brainstem rodent tumor model

Laboratory investigation

I-Mei Siu, Ph.D., Betty M. Tyler, B.A., James X. Chen, B.Sc., Charles G. Eberhart, M.D., Ph.D., Ulrich-Wilhelm Thomale, M.D., Alessandro Olivi, M.D., George I. Jallo, M.D., Gregory J. Riggins, M.D., Ph.D., and Gary L. Gallia, M.D., Ph.D.

Departments of 1Neurosurgery and 2Pathology, Johns Hopkins School of Medicine, Baltimore, Maryland; and 3Selbständiger Arbeitsbereich für Pädiatrische Neurochirurgie, Charité, Campus Virchow Klinikum, Universitätsmedizin zu Berlin, Berlin, Germany

Object. Diffuse brainstem tumors are the most difficult type of pediatric CNS malignancy to treat. These inoperable lesions are treated with radiation alone or in combination with chemotherapy, and the survival rate is less than 10%. It is therefore essential to develop a reliable animal model to screen new therapeutic agents for the treatment of this type of tumor.

Methods. A multipotent human glioblastoma stemlike neurosphere line, 060919, was established from a surgically resected glioblastoma specimen; when cells were implanted intracranially into athymic nude mice, they formed invasive, vascular tumors that exhibited the features of glioblastoma. Ten female Fischer 344 rats received an injection of 75,000 F98 rat glioma cells and 10 female athymic nude rats received an injection of 75,000 060919 human glioblastoma stemlike cells in the pontine tegmentum of the brainstem. A control group of 5 female Fischer rats received an injection of saline in the same location as the animals in the tumor groups. Kaplan-Meier curves were generated for survival, and brains were processed postmortem for histopathological investigation.

Results. Both F98 cells and 060919 cells grew in 100% of the animals injected. Median survival of animals injected with F98 was 15 days, consistent with the authors’ previous reports on the establishment of the brainstem tumor model using the F98 rat glioma line. Median survival of animals injected with 060919 was 31 days. Histopathological analysis of the tumors confirmed the presence of brainstem lesions in animals that received brainstem injections of F98 and in animals that received brainstem injections of 060919. The 060919 brainstem tumors histologically resembled glioblastoma.

Conclusions. Tumor take and median survival were consistent for animals injected in the brainstem with either the established F98 rat glioma cell line or the 060919 human glioblastoma stemlike neurosphere line. Histopathological features of the 060919 brainstem tumors resembled glioblastoma. Establishment of this human glioblastoma stemlike brainstem animal model will improve the evaluation and identification of more efficacious agents for the treatment of high-grade brainstem tumors. (DOI: 10.3171/2010.3.PEDS09366)

Key Words · brainstem · animal model · human · glioma · glioblastoma · neurospheres · stemlike cells

Diffuse pontine gliomas are the most common type of brainstem tumors and represent 10–15% of childhood CNS tumors.2 These tumors are considered inoperable because of their infiltrative nature, and treatment is limited to radiotherapy with or without adjuvant chemotherapy. The survival rate for patients with this diffuse type of brainstem tumor is dismal, and most patients usually die of this disease within 12 months of diagnosis. It is imperative to identify and test new therapeutics in an orthotopic animal model.

To test promising therapies against brainstem gliomas, it is necessary to use an animal model in which the infiltrative nature and location of the tumor are reproducible to best recreate the biology of this tumor. We have previously established brainstem animal models, using the F98 glioma and 9 L gliosarcoma lines in the syngeneic Fischer 344...
Human glioblastoma stemlike brainstem rodent model

rats.4,5 Both the F98 and 9 L lines formed highly invasive, vascular tumors in the brainstem. However, a recent study demonstrated the vast array of mutations and genomic alterations in glioblastoma,2 confirming the complex genetic nature of these tumors. Thus, we sought to establish a new brainstem tumor model using a human glioblastoma line. Previous in vivo intracranial models of glioblastoma relied upon traditional adherent glioblastoma lines. However, these adherent glioblastoma lines give rise to well-demarcated, noninvasive tumors with little vascularization.

Recently, glioblastoma stemlike neurosphere lines have been isolated and established from resected glioblastomas. These glioblastoma stemlike neurosphere lines are grown in serum-free media supplemented with the mitogens EGF and FGF-2, form continuously self-renewing stemlike neurospheres, and can be differentiated into the various neuronal and glial lineages.3,4 When implanted, these glioblastoma stemlike neurosphere lines formed invasive tumors that are histologically identical to glioblastoma.3,4 Genomic and transcript profile analysis of the original patients’ glioblastomas and the derived glioblastoma stemlike neurosphere lines revealed extensive similarities between the parent tumors and the neurosphere lines.4 However, traditional adherent lines derived from the patients’ glioblastomas were found to differ significantly from the parental tumors, both genomically and transcriptionally, suggesting that the glioblastoma stemlike neurosphere lines represent a better, more accurate model for glioblastoma.4,6 We have established a glioblastoma stemlike neurosphere line and tested its ability to form invasive, lethal glioblastomas intracranially in mice. This line, 060919, formed invasive, vascularized tumors that were histologically classified as glioblastoma. The mice, which were intracranially implanted with 060919, had a mean survival of less than a month. We then sought to establish a brainstem tumor model with 060919 using the cannulated guide screw technology with which we had established the rat glioma brainstem models.4,5

Methods

Cell Lines

Human Glioblastoma Stemlike Neurosphere Line 060919. Glioblastoma tissue was collected under an institutional-review-board–approved protocol to obtain discarded tissue. The 060919 glioblastoma stemlike neurosphere line was established from fresh surgical tissue in serum-free media supplemented with mitogens as described previously.3 Briefly, tumor resected from a patient diagnosed with glioblastoma was mechanically dissociated, passed through a 40-µm filter and transferred into complete neurosphere media, which consisted of serum-free media containing 20 ng/ml EGF and 10 ng/ml FGF-2 and maintained at 37°C and 5% CO2. Neurospheres were passaged by trituration and seeded into fresh media; the cultures were serially passaged for more than 25 passages.

The F98 Rat Glioma Cell Line. The F98 rat glioma cell line was maintained in DMEM supplemented with 10% fetal bovine serum and 100 µg/ml penicillin-streptomycin.

Flow Cytometry. The glycoprotein CD133 is thought to be a marker of glioblastoma stem cells, and thus we performed analytical flow cytometry on CD133-stained 060919 as follows: The 060919 neurospheres were triturated to obtain a single cell suspension. One hundred thousand cells were stained with either phycoerythrin-conjugated anti-CD133 antibody (Miltenyi Biotec) or with phycoerythrin-conjugated isotype control antibody (Miltenyi Biotec) following the manufacturer’s protocol and analyzed on a Beckman-Coulter FACscan.

Cell Differentiation. The differentiation potential of 060919 was ascertained as follows: Sterile 10-mm glass coverslips were placed, 1 coverslip per well, in a 24-well tissue culture plate. Diluted reduced growth factor Matrigel (1:100, BD Biosciences) was added to each well, and the plate was placed in the cell culture incubator overnight. The next day, 060919 neurospheres were triturated to obtain a single cell suspension and were then plated onto the Matrigel-coated coverslips at a concentration of 5000 cells per well in media identical to complete neurosphere media except for the absence of EGF. After 48 hours, the media was replaced with neurosphere media that lacked both EGF and FGF-2. The cells were then maintained in this media for 7 days until immunofluorescent staining for markers of differentiation.

Immunofluorescent Staining. The differentiation status of 060919 was then assessed by immunofluorescent staining after differentiation as previously described with some modifications.3 Immunostaining was performed on the coverslips in a 24-well plate. Briefly, cells were washed twice with cold PBS and fixed in ice-cold 4% paraformaldehyde for 30 minutes at room temperature. The cells were then washed twice with PBS/0.1% Triton and blocked for 1 hour at room temperature with 10% goat serum/PBS. After removal of the blocking solution, cells were incubated overnight at 4°C with antibodies against either GFAP (rabbit polyclonal, 1:500, Dako) or Tuj1 (mouse monoclonal, 1:500, Chemicon/Millipore). The next day, cells were washed 3 times with PBS/0.1% Triton and incubated with goat anti–rabbit antibody conjugated to Alexa 488 (1:500, Molecular Probes/Invitrogen) for GFAP or goat anti–mouse antibody conjugated to Alexa 594 (1:500, Molecular Probes/Invitrogen) for Tuj1. The antibodies were removed and cells were washed 3 times with PBS/0.1% Triton, after which the coverslips were mounted on slides using Vectashield-DAPI mounting medium (Vector Laboratories) and visualized with a fluorescence microscope (Model No. IX81, Olympus America, Inc.).

Intracranial Xenografts. For this study, 5- to 6-week-old female athymic nude mice (NCI-Frederick) were used. The mice were housed in standard facilities and given free access to Baltimore City water and mouse chow. The experimental protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University and met all federal guidelines. Two groups of mice received intracranial implantations of 100,000 (3 mice) and 500,000 (3 mice) freshly dissociated 060919.
cells as follows: Each animal was anesthetized by intraperitoneal injection with 0.15 ml of stock solution of ketamine (Abbott Laboratories) and xylazine (Phoenix Pharmaceutical), 25 mg/kg and 2.5 mg/kg, respectively; for each animal, a midline incision was made and a bur hole was drilled 1 mm posterior and 3 mm lateral to the bregma over the parietal lobe using a 2-mm drill bit and a dental drill without disrupting the dura mater. After the dura was exposed, the animal was placed in a stereotactic frame and a needle containing the tumor cells was placed into the brain at a depth of 2.5 mm. The solution of cells (2 µl) was delivered with a 22-gauge 10 µl Hamilton needle (Hamilton Co.) over 1 minute, after which the needle was removed and the area washed with sterile saline. The wound (approximately 1 cm long) was then closed with sterile autoclips.

**Brainstem Experimental Design**

**Animals.** For this study, female Fischer 344 rats (Charles River Laboratories) weighing 170–200 g (average weight, 189 g) and female athymic nude rats (NCI-Frederick) weighing 217–275 g (average weight, 242 g) were used. The rats were housed in standard facilities and given free access to Baltimore City water and rat chow. The experimental protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University and met all federal guidelines.

**Experimental Groups.** Female Fischer F344 rats (10 rats) received a 3-µl injection of 75,000 cells total of the syngeneic F98 rat glioma cells. Female athymic nude rats (16 rats) were randomized into 2 groups: the first group (5 rats) received a 3-µl injection of saline and the second group (10 rats) received a 3-µl injection of 75,000 cells of the 060919 cell line. An eleventh athymic nude rat received an injection of 1000 cells of the 060919 line. The injections for all 3 groups were at the same coordinates.

In a pilot experiment, several female athymic nude rats received 80,000 cells of the 060919 line in the brainstem and were killed 15 days postimplantation to identify the injection site and ascertain whether tumor had established itself within the brainstem.

**Surgical Technique.** Brainstem injections were performed as previously described.63 Briefly, animals were anesthetized with intraperitoneal injections of a solution of ketamine 50 mg/kg (Abbott Laboratories) and xylazine 10 mg/kg (Phoenix Pharmaceutical). The cranial regions of the Fischer 344 rats and the athymic nude rats were prepared in a sterile fashion, and in the Fischer 344 rats, the cranial regions were shaved first. A midline incision approximately 2-cm in length was made in the skin over the skull and, using a steel drill bit (Plastics One), a small bur hole was made at coordinates 1.4 mm right of the sagittal suture and 1.0 mm anterior to the lambdoid suture. Cannulated guide screws (Plastics One) were placed in the bur holes, and the animals were then positioned +45° from the horizontal plane. A 22-gauge 10 µl-Hamilton needle was inserted to a depth of 7 mm from the dura mater, and then 3 µl of saline, F98 cells, or 060919 cells were injected into the brainstem. The incision was closed with surgical staples in a standard fashion and the animals were allowed to recover before being returned to their cages.

**Histological Studies.** The animals were weighed every other day until death, at which point the entire brain was dissected en bloc and fixed in 10% formalin for 24 hours before histological processing. The brains were sectioned, stained with H & E, and analyzed by a neuropathologist (C.G.E.).

**Statistical Analysis.** Kaplan-Meier survival curves for the 3 groups were generated using the log-rank test with the statistical software package GraphPad Prism, v. 4 (GraphPad Software).

**Results**

**Establishment and Characterization of 060919 Glioblastoma Neurosphere Line**

Tumor tissue was obtained from a patient whose MR image is shown in Fig. 1A. We processed the tumor as described above and the resulting cell line, 060919, grew as a suspension culture of self-renewing stemlike neurospheres (Fig. 1B). When implanted intracranially into immunocompromised mice, this neurosphere line formed invasive, highly vascularized tumors (Fig. 1E and F) with histopathological features consistent with the original tumor. The differentiation of 060919 into astrocytes (Fig. 1C, green fluorescent cells) and neurons (Fig. 1C, red fluorescent cells) is shown in Fig. 1. Analytical flow cytometry of the 060919 stemlike glioblastoma neurosphere line revealed that 060919 is approximately 60% CD133-positive (Fig. 1D).

**Survival Following Injection Into the Brainstem**

The median survival for the Fischer 344 rats implanted with 75,000 of the F98 rat glioma cells in the brainstem was 15 days. The median survival for the athymic nude rats implanted with 75,000 of the 060919 human glioblastoma stemlike neurosphere cells in the brainstem was 31 days (Fig. 2). The athymic nude rat implanted with 1000 cells was not included in this curve, as it was not implanted with the same number of cells as the other rats; however, this animal died of tumor 48 days postimplantation. None of the animals injected with saline died during this study.

**Histopathological Examination**

The histopathological examination results of the animals injected with F98 rat glioma cells were consistent with those described previously;69 briefly, the tumors were highly infiltrative and exhibited limited angiogenesis. The histopathological examination of the animals injected with 060919 human glioblastoma stemlike neurosphere cells revealed infiltrative lesions (Figs. 3A and C and Fig. 4) that exhibited a high degree of mitotic activity (Fig. 3D) and contained neoplastic giant cells (Fig. 3B). These lesions also exhibited a moderate degree of angiogenesis (Fig. 4).
Human glioblastoma stemlike brainstem rodent model

Discussion

Confirmation of the F98 Brainstem Tumor Model

The F98 rat glioma cell line is a standard rat brain tumor model that forms infiltrative tumors of moderate angiogenic activity in syngeneic animals. Previously, we used the F98 cell line to develop a novel brainstem tumor model system.4,5 The advantages of this model are the reproducibility of tumor establishment and growth, the exhibition of the classical histological features of diffuse brainstem tumors by the resulting lesions, and the extensive characterization of this line. This model system has been used for in vivo testing of chemotherapeutics and delivery systems, including convection-enhanced delivery. Moreover, this line confers the ability to investigate immune-based therapies in an immunocompetent animal model.

Establishment of a Human Glioblastoma Stemlike Brainstem Tumor Model

One limitation of the F98 and other brainstem tumor models, however, is the use of nonhuman cell lines. Recently, stemlike cells were isolated from glioblastoma; these grow as self-renewing neurospheres in serum-free media supplemented with the mitogens EGF and FGF-2.3 When injected intracranially, these glioblastoma stemlike neurospheres form highly invasive tumors that exhibit the clinical features of glioblastoma.3 We thus sought to establish a brainstem animal model using a human glioblastoma
I. M. Siu et al.

stemlike neurosphere line. This line, 060919, forms invasive, vascular tumors with histological features consistent with glioblastoma in athymic rodents. This glioblastoma stemlike neurosphere line also exhibits stemlike characteristics associated with previously reported glioblastoma stemlike neurosphere lines, such as the ability to form self-renewing neurospheres, the potential to differentiate into glial and neuronal lineages, and the expression of high levels of CD133.

Given the genetic complexity of human glioblastoma tumors, we feel this line will serve as a better model system than nonhuman cell lines. The median survival of animals that received brainstem injections of cells from the 060919 line was 31 days, which is longer than that of animals which received F98 cells (median survival 15 days). We believe this increase is not an impediment to chemotherapeutic studies, as other human glioblastoma lines we have tested had much longer median survival times (data not shown). This might also allow a longer window for experimental treatment regimens. The need to identify more efficacious therapies for this invariably fatal disease may be better addressed by the use of human glioblastoma stemlike lines instead of nonhuman glioma lines.

We have established the 060919 glioblastoma stemlike neurosphere line from resected tissue, and this line grew in 100% of the animals injected intracranially as well as in the brainstem. One limitation of this study is that this cell line was derived from an adult patient with a temporal lobe glioblastoma, and a better model system may be derived from a brainstem glioma in a pediatric patient. These patients, however, often do not undergo surgery and if they do, the procedure is usually a biopsy with limited tissue being obtained. Nonetheless, we feel this system represents a better model than the currently used nonhuman cell line–based models. Additionally, it has also been shown that glioblastoma stemlike neurosphere lines are more closely related genetically to the original tumors obtained from patients than are traditional adherent glioblastoma cell lines. Our hope is that the establishment of this human stemlike cell model will lead to the identification of novel and more efficacious chemotherapeutic compounds for the treatment of this invariably fatal disease by closely representing a human glioblastoma.

Conclusions

We have established a new reproducible brainstem animal model based on a human glioblastoma stemlike neurosphere line, 060919. When injected into the brainstems of athymic nude rats, 060919 gave rise to infiltrative, actively proliferating tumors that were histologically identical to human glioblastoma. Tumor take for the 060919 neurosphere line was 100%, and median survival was 31 days. The establishment of this brainstem tumor model represents an improvement over other models that are based on nonhuman cell lines.

Disclosure

This work was supported by funding from the Brain Tumor Funders’ Collaborative to Dr. Riggins.

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this
Human glioblastoma stemlike brainstem rodent model

paper. Author contributions to the study and manuscript preparation include the following. Conception and design: Gallia, Siu, Jallo. Acquisition of data: Gallia, Siu, Tyler, Chen, Thomale, Jallo. Analysis and interpretation of data: Gallia, Siu, Chen, Eberhart, Olivi, Jallo, Riggins. Drafting the article: Siu. Critically revising the article: all authors. Reviewed final version of the manuscript and approved it for submission: all authors. Statistical analysis: Gallia, Siu.

References


Address correspondence to: Gary L. Gallia, M.D., Ph.D., Department of Neurosurgery and Oncology, Johns Hopkins Hospital, Baltimore, Maryland 21287. email: ggallia1@jhmi.edu.