Animal model of intramedullary spinal cord glioma using human glioblastoma multiforme neurospheres

Laboratory investigation

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Objective. Advances in the diagnosis and management of patients with spinal cord tumors have been limited because of the rarity of the disease and the limitations of current animal models for spinal cord glioma. The ideal spinal cord tumor model would possess a number of characteristics, including the use of human glioma cells that capture the growth pattern and local invasive nature of their human counterpart. In this study, the authors’ goal was to develop a novel spinal cord tumor model using a human neurosphere cell line.

Methods. Eighteen female athymic rats were randomized into 3 experimental groups. Animals in the first group (6 rats) received a 3-ml intramedullary injection containing DMEM and were used as controls. Animals in the second group (6 rats) received a 3-ml intramedullary injection containing 100,000 glioblastoma multiforme (GBM) neurosphere cells in 3 ml DMEM. Animals in the third group (6 rats) received a 3-ml intramedullary injection containing 9L gliosarcoma cells in 3 ml DMEM. Functional testing of hindlimb strength was assessed using the Basso-Beattie-Bresnahan (BBB) scale. Once the functional BBB score of an animal was less than or equal to 5 (slight movement of 2 joints and extensive movement of the third), euthanasia was performed.

Results. Animals in the GBM neurosphere group had a mean survival of 33.3 ± 2.0 days, which was approximately twice as long as animals in the 9L gliosarcoma group (16.3 ± 2.3 days). There was a significant difference between survival of the GBM neurosphere and 9L gliosarcoma groups (p < 0.001). None of the control animals died (p < 0.001 for GBM neurosphere group vs controls and 9L vs controls). Histopathological examination of the rats injected with 9L gliosarcoma revealed that all animals developed highly cellular, well-circumscribed lesions causing compression of the surrounding tissue, with minimal invasion of the surrounding gray and white matter. Histopathological examination of animals injected with GBM neurospheres revealed that all animals developed infiltrative lesions with a high degree of white and gray matter invasion along with areas of necrosis.

Conclusions. The authors have established a novel animal model of spinal cord glioma using neurospheres derived from human GBM. When injected into the spinal cords of athymic nude rats, neurospheres gave rise to infiltrative, actively proliferating tumors that were histologically identical to spinal cord glioma in humans. On the basis of their results, the authors conclude that this is a reproducible animal model of high-grade spinal cord glioma based on a human GBM neurosphere line. This model represents an improvement over other models using nonhuman glioma cell lines. Novel therapeutic strategies can be readily evaluated using this model.

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Key Words • animal model • glioblastoma • neurosphere • spinal cord • stem cell • oncology

The management of patients with ISCGs continues to be a challenge for neurosurgeons and oncologists. The prognosis of ISCGs remains poor due to their infiltrative nature, high recurrence rate, and limited treatment options.1 These lesions represent 5%–25% of all intraspinal tumors and are more prevalent in children than in adults.9 Resection is the standard of care for these patients,10,11,16–18 particularly for patients with low-grade gliomas. However, this can be difficult given the infiltrative nature of ISCGs, the presence of surrounding normal cord, and the absence of clear surgical planes between tumor and normal spinal cord tissue.11

The use of radiotherapy or chemotherapy for the treatment of ISCGs remains controversial. Radiotherapy is often used as an adjunct to resection for high-grade tumors.17

Abbreviations used in this paper: BBB = Basso-Beattie-Bresnahan; EGF = epidermal growth factor; FGF = fibroblast growth factor; GBM = glioblastoma multiforme; ISCG = intramedullary spinal cord glioma.

This article contains some figures that are displayed in color online but in black and white in the print edition.
The reported efficacy of this strategy, however, varies significantly between centers and is influenced strongly by tumor grade.\(^10\) The 10-year survival rates for patients with low-grade astrocytomas receiving postoperative radiation therapy range from 40% to 91%.\(^5,12,14,19\) In contrast, the majority of institutional series report that no patient with a high-grade spinal cord astrocytoma survives after 2 years.\(^8,12,14,15\) At present, there is no clearly defined chemotherapeutic regimen for spinal cord gliomas beyond the context of clinical trials.

Advances in the diagnosis and management of patients with spinal cord tumors have been limited due to the rarity of the disease and the limitations of current animal models of spinal cord glioma. The ideal spinal cord tumor model would possess a number of characteristics, including the use of human glioma cells that capture the growth pattern and local invasive nature of their human counterpart. We have previously reported a novel model of ISCG utilizing rat glioma cell lines injected into the spinal cords of immunocompetent Fischer rats.\(^4\) Although highly reproducible, this model utilizes nonhuman glioma cell lines that may fail to accurately reproduce the complex tumor biology of human spinal cord glioma. Although the use of these murine glioma cell lines for intracranial models of glioma is well accepted and is the foundation for many in vivo studies, it is unclear if this holds true for modeling human spinal cord tumors.

Recently, cancer stem-like cell lines have been isolated and established from resected GBM. These GBM cancer stem-like cells are grown in serum-free media supplemented with the mitogens EGF and FGF-2 to form continuously self-renewing neurospheres (Fig. 1) that can be differentiated into the various neuronal and glial lineages.\(^8,13\) When implanted, these GBM neurospheres formed invasive tumors that were histologically identical to GBM.\(^8,13\) Subsequent genomic and transcript profile analysis of the patient’s original GBM tissue and the derived GBM neurosphere cell line demonstrated remarkable similarity.\(^13\) However, a traditional adherent cell line derived from the patient’s GBM tissue differed significantly from the parent tumor both genomically and transcriptionally, thus suggesting that the GBM neurosphere cell line represents a more accurate model for GBM.\(^13\)

We have previously established a GBM neurosphere line and tested its ability to form invasive, lethal GBMs intracranially in the brainstems of mice. This line (060919) formed invasive, vascularized tumors which were histologically identical to human GBM.\(^21\) In this study, we describe a novel spinal cord tumor model using this novel human neurosphere cell line.

### Methods

Eighteen female athymic rats (Harlan Laboratories, Inc.) weighing 150–200 g were randomized into 3 experimental groups. Animals in Group 1 (6 rats) received a 3-μl intramedullary injection containing DMEM and were used as controls. Animals in Group 2 (6 rats) received a 3-μl intramedullary injection containing 100,000 GBM neurosphere cells in 3 μl DMEM. Animals in Group 3 (6 rats) received a 3-μl intramedullary injection containing 9L gliosarcoma cells in 3 μl DMEM. The hindlimb motor function of the animals was assessed as described below, and the rats were killed after the onset of paraparesis for histopathological analysis. They were housed in standard facilities and were given free access to water and rodent chow. All of the rats were treated in accordance with the policies and principles of laboratory animal care of the Johns Hopkins University School of Medicine Animal Care and Use Committee.

### Tumor Lines

**Human GBM Neurosphere Line 060919.** Human GBM tissue was collected under an institutional review board–approved protocol to obtain discarded tissue. The 060919 GBM stem-like neurosphere line was established from fresh surgical tissue obtained from a patient with a GBM in serum-free media supplemented with mitogens as described previously.\(^8\) Briefly, tumor resected from a patient diagnosed with a GBM was mechanically dissociated, filtered, and transferred into complete neurosphere media (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.

**9L Gliosarcoma.** The 9L gliosarcoma was obtained from Dr. M. Barker at the University of California, San Francisco Brain Tumor Research Center. The cell lines were grown in DMEM (Gibco, Invitrogen Corp.) with 4.5 g/L glucose, supplemented with 10% fetal bovine serum and penicillin/streptomycin. A tumor suspension was prepared by suspending 100,000 cells in 5 ml DMEM.

### Surgical Technique

The rats were anesthetized with an intraperitoneal injection (0.4–0.6 ml) of a stock solution containing ket-
amino hydrochloride (25 mg/ml) (Hospira, Inc.), xylazine (2.5 mg/ml, Phoenix Pharmaceutical, Inc.), and 14.25% ethanol in normal saline. The rats were placed on a sterile field, and their backs were shaved and prepared with a Betadine solution. The spinous processes in the midthoracic region were identified, and a 2-cm longitudinal incision was made over the dorsal midthoracic region. The underlying fascia and the paravertebral muscles were retracted laterally, a single spinous process was removed with a rongeur, and the ligamentum flavium was removed to expose the dura. Three microliters of DMEM (Group 1) or 3 μl DMEM with 100,000 neurosphere cells (Group 2) was injected through the dura with the aid of a 26-gauge Hamilton syringe (Hamilton Co.). The needle was advanced until the dorsal aspect of the vertebral body was encountered. Penetration of the spinal cord was confirmed by monitoring a lower-extremity motor reflex after needle insertion. Injection occurred over the course of 1 minute to minimize extravasation of cells from the spinal cord. The wounds were closed with surgical staples, and analgesia was provided with an intraperitoneal injection of 0.2 mg of buprenorphine (Abbott Laboratories).

**Functional Testing**

Functional testing of hindlimb strength was assessed using the BBB scale. Briefly, the rats were placed in an open-field testing area. Once the rat walked continuously, it was observed for 4 minutes and locomotion was rated using the BBB locomotor scale. The BBB scale is a 21-point scale ranging from 21 (consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability, and tail consistently up) to 0 (no observable hindlimb movement). All animals were tested preoperatively to ensure a baseline locomotor rating score of 21. Postoperatively, the rats were tested once every 1–2 days.

**Euthanasia**

Once the functional BBB score of an animal was less than or equal to 5 (slight movement of 2 joints and extensive movement of the third), euthanasia was performed by CO₂ overexposure. All animals surviving 60 days would be killed.

**Histopathological Analysis**

After the rats were killed, the entire spinal cord was removed en bloc from the spinal canal, and a segment encompassing all macroscopically visible tumor was excised and placed in 4% formalin in phosphate-buffered saline. Axial sections (2 mm each) were obtained after fixation and embedded in paraffin. Five slides (10-mm thick) were obtained from each section for H & E staining. Immunohistochemical staining was conducted using the avidin-biotin-peroxidase complex method according to recommendations by the manufacturers. Glial fibrillary acidic protein polyclonal antibody (Dako) at a 1:100 dilution was used to further identify the astrocytic lineage. Histopathological analysis was performed using a Zeiss optical microscope at standard magnifications.

**Statistical Analysis**

In this study, a BBB functional score of less than or equal to 5 was the primary end point. Survival times were compared between groups using the log-rank (Mantel-Cox) test in Kaplan-Meier nonparametric analysis of survival. Statistical software (version 8.0, for Windows; SPSS, Inc.) was used for the statistical analyses. The results for survival are reported as the mean ± standard deviation.

**Results**

**Functional Progression**

Animals in the GBM neurosphere group had a mean survival of 33.3 ± 2.0 days, which was approximately twice as long as animals in the 9L gliosarcoma group (16.3 ± 2.3 days). There was a significant difference between survival of the GBM neurosphere and 9L gliosarcoma group (p < 0.001). None of the control animals died (p < 0.001 for GBM neurosphere group vs controls and 9L vs controls) (Fig. 2).

**Histopathological Examination**

Examination of the control animals did not reveal significant findings. Histopathological examination of those animals injected with 9L gliosarcoma revealed that all animals developed highly cellular, well-circumscribed lesions causing compression of the surrounding tissue with minimal invasion of the surrounding gray and white matter (Fig. 3).

Histopathological examination of the rats injected with GBM neurospheres revealed that all animals developed infiltrative lesions with a high degree of white and gray matter invasion along with areas of necrosis (Fig. 4). All tumors appeared to originate from the intramedullary tissue (rather than the extramedullary space), as all specimens had a clear surrounding border of normal gray/white matter. It is possible that during the initial injection of neurospheres or during subsequent tumor growth, tumor cells may have migrated along the subarachnoid space to distant extramedullary sites along the spinal cord. Although there

**Fig. 2.** Graph showing the time to paraplegia after spinal cord injection. Animals in the GBM neurosphere group (NS) had a mean survival of 33.3 ± 2.0 days, which was approximately twice as long as animals in the 9L gliosarcoma group (16.3 ± 2.3 days). There was a significant difference between survival of the GBM neurosphere and 9L gliosarcoma groups (p < 0.001).
was no evidence of large sites of distant tumor growth upon gross examination of the spinal cord, the possibility of microscopic sites of tumor cannot be excluded. Intense immunoreactivity for glial fibrillary acidic protein was readily seen in 9L tumors, confirming their glial nature. Animals injected with GBM neurospheres showed significantly less glial fibrillary acidic protein immunoreactivity than the 9L-injected animals, suggesting a more de-differentiated state.

### Discussion

The 9L and F98 rat glioma cell lines have been used to establish standard brain tumor models in rats that form infiltrative tumors with low immunogenic potential and moderate angiogenic activity in syngeneic animals. Pre-}

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**Fig. 3.** Photomicrographs of an axial section of a rat spinal cord after injection of 9L gliosarcoma cells. A well-circumscribed fibrous mass is seen in the dorsal white matter (A). The tumor exhibits islands of high cellularity with multiple areas of necrosis (B–D). Invasion of the white matter tracts with expansion of the fibrotic capsule is consistent with the sarcomatous phenotype of the 9L cell line. H & E.

**Fig. 4.** Photomicrographs of an axial section of a rat spinal cord after injection of 100,000 cells. An infiltrative lesion with high cellularity and atypical cells with multiple mitotic figures can be observed. Cellular clustering around dysmorphic vascular channels results in a papillary architecture with diffuse infiltration of gray and white matter and destruction of normal cord structures. H & E.
a functional immune system. Second, our animal model uses neurospheres derived from intracranial GBM rather than spinal cord glioma. It is unclear whether the differences between high-grade glioma in the brain and spinal cord would potentially affect the validity of our model. Finally, the cells used display an aggressive growth pattern in vivo relative to the growth pattern typically observed in low-grade spinal cord glioma in humans. It is unclear if therapeutic strategies that show efficacy in our spinal cord model would be successful in patients with low-grade, rather than high-grade, spinal cord glioma.

Conclusions

We have established a novel animal model of spinal cord glioma based on the human GBM neurosphere line 060919. When injected into the spinal cords of athymic nude rats, 060919 gave rise to infiltrative, actively proliferating tumors that were histologically identical to spinal cord glioma in humans. On the basis of our results, we conclude that this is a reproducible animal model of high-grade spinal cord glioma based on a human GBM neurosphere line. This model represents an improvement over other models in which nonhuman glioma cell lines have been used. Novel therapeutic strategies may potentially be evaluated using this model.

Disclosure

Dr. Gokaslan has direct stock ownership in US Spine and Spinal Kinetics. He received clinical or research support for the study described from AO Spine, Medtronic, DePuy, and NREF.

Author contributions to the study and manuscript preparation include the following. Conception and design: Hsu, Siu, Pradilla, Jallo, Gallia. Acquisition of data: Pradilla. Analysis and interpretation of data: Hsu, Gokaslan. Drafting the article: Hsu. Critically revising the article: Hsu, Siu, Pradilla, Jallo, Gallia. Reviewed submitted version of manuscript: Hsu, Siu, Pradilla, Jallo, Gallia. Approved the final version of the manuscript on behalf of all authors: Hsu. Statistical analysis: Hsu. Administrative/technical/material support: Hsu. Study supervision: Hsu, Jallo, Gallia.

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