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Rodent Glioma Models: Intracranial Stereotactic Allografts and Xenografts

Hikmat Assi¹, Marianela Candolfi¹, Pedro R. Lowenstein¹, Maria G. Castro¹

¹Department of Neurosurgery and Department of Cell and Developmental Biology, University of Michigan Medical School, 4570 MSRB II, 1150 West Medical Center Drive, Ann Arbor, MI, 48109, USA

Abstract

Modeling human disease in small animals has been fundamental in advancing our scientific knowledge and for the development of novel therapeutic strategies. In the case of brain cancer, implantable tumor models, both intracranial and also in the periphery, have been widely used and extensively characterized. These models can be used to better understand certain aspects of tumor biology such as growth, neovascularization, response to potential therapies, and interaction with the immune system. Brain tumors from patients as well as rodents have been cultured in vitro, in an attempt to establish permanent cell lines. Human glioma tumors have also been maintained by serial passage in the flanks of immune-deficient animals, as it has been shown that it is not feasible to continuously passage them in culture. In this chapter, we describe various gliomas that have been isolated from mice, rats, and humans and subsequently used as syngeneic or xenograft tumor models in vivo. The majority of the models that we present in this chapter arose either spontaneously or by administration of chemical carcinogens. We compare and contrast the histopathological, genetic, and invasive features of the tumor lines as well as identify novel treatment modalities that have been developed. Finally, we present the procedures for intracranial implantation of tumor cells in rodents using stereotactic surgical techniques. The use of this technique enables the generation of large numbers of animals harboring intracranial tumors with relative ease and the survival of tumor-bearing animals is highly reproducible. These characteristics make the use of these in vivo models very attractive when aiming to develop and test the effectiveness of novel anticancer therapies.

Keywords

Xenograft; Allograft; Stereotactic; Glioma models; Brain cancer; Neurosurgery in rodents; Tumor implantation

*Corresponding Author: Maria G. Castro, mariacas@med.umich.edu.

1. Introduction

1.1. Brain Tumor Models

Human gliomas arise from glial cells and although rare, they are extremely malignant. Patients diagnosed with glioblastoma multiforme (GBM), the most common and aggressive form of brain tumors, face a high mortality rate (1). The highly invasive nature of CNS tumors as well as their anatomical location makes complete resection difficult if not impossible. Therefore, the preclinical development of novel therapies requires stringent brain cancer models that replicate the salient features of the human disease. Ideally, small animal models should retain the important features of human gliomas such as growth patterns, neuropathological features, vascularization, and genetic alterations to be considered useful models of brain cancer. These models must also be relatively easy to generate and highly reproducible. While various tumor models have been developed, there are none that perfectly emulate the human disease. Traditionally, tumors of the CNS have been generated by administration of carcinogenic chemicals such as *N*-ethyl-*N*-nitrosourea (ENU) and *N*-methyl-*N*-nitrosourea (MNU) (2–4). Currently, there are two main methods to develop brain tumor models: endogenous and transplantable tumor models. Endogenous brain tumors can be induced by intracranial administration of oncogenic viruses (5–7). Astrocytic brain tumors have been developed after the administration of vectors encoding Ras and AKT (8), EGFR or PDGF-B (9) into the brain of mice that are deficient in PTEN, Ink4a/Arf knockouts, or p53 (10, 11). Administration of retroviral vectors expressing PDGF-B also generates gliomas in rats (5). In all of these models, tumors appear within 3–12 months posttumor induction. These endogenous models exhibit the histopathological features and contain the genetic alterations of human GBM, constituting unique tools to study gliomagenesis. However, the variable latency and the fact that not all the animals develop tumors remain obstacles to assess the efficacy of novel therapeutic approaches using these endogenous models. Recently, a novel approach to induce endogenous GBM that uses the *Sleeping Beauty* (SB) transposable element has been described (12). This technique allows the integration of several oncogenes in cells of the mouse brain (13). Spontaneous brain tumors displaying the histological characteristics of human GBM were induced by injecting a DNA plasmid encoding SB-transposase in combination with plasmids containing the oncogenes AKT, NRAS, or EGRFvIII flanked by transposase recognition elements into the brain of neonatal mice (13). As a result, the mice developed tumors in roughly 3 weeks that mimic the genetic heterogeneity found in patients and structurally resembled human astrocytomas or glioblastomas.

Since transplantable rodent GBM models are technically easy to develop and very reproducible, they constitute a unique tool to evaluate novel therapies in vivo. In this protocol, we present several mouse, rat, and human GBM tumors that scientists have cultured in vitro, in an attempt to establish cell lines which have then been used to generate transplantable orthotopic glioma models (Figs. 1–3). Human GBM xenografts implanted in the brain of nude mice allow studying the response of human GBM cells to antglioma approaches, such as cytotoxic and antiangiogenic agents in vivo in the context of the brain (14–18) and retain many of the genetic alterations present in the original specimen (19–21). However, due to the lack of an intact immune system, this model is not suitable to assess

antitumor immune responses and tumor–host interactions. On the other hand, although syngeneic tumor models are useful tools to test therapeutic approaches that stimulate the host immune response against the tumor, their anatomical pattern of invasion and genetic profile differs from human GBM. Thus, it is advisable to use several models to corroborate preclinical findings (22–24).

Stereotactic surgery is a well-established technique for targeting precise anatomical structures within the brain. This technique can be used to implant glioma cells in the brain and quickly generate tumor-bearing animals to study various aspects of neuro-oncology, such as angiogenesis, invasiveness, tumor-induced immune suppression, and efficacy of novel therapeutics. This protocol will describe how to perform intracranial implantation of cultured tumor cells in mice and rats using a stereotactic apparatus as well as the steps necessary for maintaining primary human tumor cultures.

2. Materials

1. Glioma cell line of interest resuspended PBS or serum-free media (injectable volume ~1–5 µl). Cells can be kept for 2–3 h on ice during surgery.
2. Adult rat (250 g body weight. Fisher or Lewis Strain. Charles River Inc., <http://www.criver.com>).
3. Adult female mouse (20–25 g weight. 6–8 weeks old C57Bl/6, VM/Dk, Rag1^{−/−} strain, Athymic Balb/c. Jackson Labs Inc., <http://www.jax.org>).
4. Drugs:
 - a. Ketamine (Bioniche Teoranta, <http://www.bionichepharmausa.com>)
 - b. Dexmedetomidine (Orion Corp., <http://www.orion.fi>)
 - c. Carprofen (Hospira Inc., <http://www.hospira.com>)
 - d. Xylazine (Animal Health International, <http://www.pvpl.com>)
 - e. Atipamezole (Orion Corp., <http://www.orion.fi>)
 - f. Buprenorphine (Animal Health International, <http://www.pvpl.com>)
5. Betadine (Bruce Medical, cat# FR-2100–88).
6. Puralube: Ophthalmic ointment (Amazon Inc., <http://www.amazon.com>).
7. Warm Lactated Ringer's solution (Hospira Inc., cat# 0409-7953-30).
8. Tissue culture:
 - a. Accutase™ Enzyme Cell Detachment Medium (eBiosciences Inc., cat# 00–4555-56)
 - b. Dulbecco's PBS, pH 7.4, without Ca or Mg (MediaTech Inc., cat# 21–040-CM)

- c. Dulbecco's modified Eagles media (DMEM), supplemented with 10% fetal bovine serum (MediaTech Inc., cat# 10-017-CM and Omega Scientific, cat# FB-01)
 - d. T75 Flasks (VWR, cat# 82050-856)
 - e. Matrigel™ (BD Pharmingen, cat# 356231)
 - f. Hemocytometer (VWR, cat# 15170-263)
- 9. Stereotactic frame with rat and mouse adapters and blunt ear bars (Stoelting Inc.).
- 10. Stereomicroscope (e.g., Zeiss Stemi 1,000 zoom) equipped with 16× eyepieces and 0.4× auxiliary objective lens, and mounted on hinged coupling arm on a heavy foot stand (or equivalent).
- 11. Electric drill with 1.75-mm and 0.6-mm drill bit (Stoelting Inc., cat# 58610).
- 12. Digital scale for animal weight (Harvard Apparatus, cat# 724586).
- 13. Fiber-optic illuminator with twin goose-neck pipes (Leica Inc.).
- 14. 10-μl, 26-G Hamilton syringe with needle (Fisher, cat# 701RN).
- 15. 5–10 μl, 33-G Hamilton syringe with needle (Fisher, cat# 75RN).
- 16. 3-0 Nylon sutures (Ethicon Inc., cat# 1663H).
- 17. Petri dish, plastic (BD Pharmingen, 353002).
- 18. Surgical equipment:
 - a. Surgical shavers (Stoelting Inc., cat# 58610)
 - b. Scalpel and blades (Cardinal Health, cat# D2862-15)
 - c. Skin retractors (Fine Science Tools Inc., mouse cat# 17000-03, rat cat# 17000-04)
 - d. Cotton swabs (VWR, cat# 89031-270)
 - e. Curved and straight forceps
 - f. Holding scissors
 - g. Sharp scissors
 - h. Sterile gauze (VWR, cat# 95038-720)
 - i. Bead sterilizer (VWR, cat# IS-350)
 - j. Red lamp (Sylvania, cat# 14663)
 - k. Optional: infant incubator for recovery of nude mice

3. Methods

3.1. Glioma Cell Lines: Maintenance and Passage

Most of the tumor cell lines presented in the chapter (Figs. 1–3) Maintenance and can be passaged indefinitely in tissue culture and cryopreserved for Passage long-term storage with the exception of GBM12. While the genetic features of the original human GBM specimen are maintained with serial tumor passage in vivo (19, 25), GBM12 cell passage in vitro for long periods of time results in loss of EGFR amplification and gain of MGMT promoter methylation (26). Thus, this human tumor must be serially transplanted in the flanks of immune-deficient mice, such as Rag1^{-/-} mice or athymic nude mice. Short-term GBM12 cultures can be derived from flank tumor in order to perform in vitro studies or to establish intracranial tumors (26). To achieve highly reproducible intracranial GBM12 xenografts, it is necessary to implant the same number of cells in the brain of all the animals. Short-term GBM12 cells growing in a monolayers can be harvested and counted for intracranial implantation (see below) (19, 26, 27).

1. Swab the skin with Betadine to minimize the risk of infection and excise the tumor from the flank of the mouse using a sterile scalpel and place in a Petri dish with 4 ml of DMEM.
2. Mince the tumor into small 1–2 mm pieces using razor blade until tissue can pass through a 1 cc syringe. Draw up and expel the tissue through the syringe to break up the tumor into even smaller pieces.
3. At this point, the cells can be cultured in vitro or injected into the flank of new RAG1-deficient mice for in vivo propagation. Short-term cultures can be used for in vitro experiments or in preparation for intracranial implantation. Minced tissue preparations are difficult to count and inject accurately, therefore, they must be cultured in vitro first. GBM12 cultures can be harvested using trypsin and cells can be counted and resuspended in the concentration required to yield accurate survival curves when implanted in the brain (19, 26, 27).
4. To culture the cells, collect and seed in MatrigelTM-coated T75 flasks with DMEM containing 10% fetal calf serum. Cells grow in monolayer and can be passaged for up to 1 month before injecting them into the brain. Their ability to grow in vivo decays and the genetic alterations present in the original human GBM specimen are lost when maintained in culture for longer periods of time.
5. Following short-term culture (2–3 weeks), the cells are harvested with AccutaseTM Detachment Medium. To prepare cells for injection, obtain a count using a hemocytometer and resuspended in DMEM (serum free) at 1×10^5 cells/ μ l (3 μ l/mouse).
6. To propagate the tumor, transfer the minced tissue into a conical tube and allow the material to settle. Remove the excess media and add MatrigelTM to the wet tissue in a 1:1 ratio.
7. 200 μ l of this mixture is injected into the flank of 2–3 immune-deficient mice using 1 cc syringes with 21 G needle. Once the tumors reach 1–2 cm in diameter,

usually 1–2 months after tumor implantation, the animal is sacrificed and the flank tumor is excised to be re-implanted in the flank or cultured in vitro.

3.2. Glioma Models: Intracranial Grafts in Rodents

3.2.1 Animals and Surgical Preparation

1. Prior to performing any procedures involving an animal, the Intracranial Grafts operator should ensure that the surgical area is clean, well in Rodents organized, and contains all required instruments. Tools can be sterilized by placing them in a flash bead sterilizer for a short time. This sterilization technique is sufficient for rodent surgery but not for larger animals.
2. Lay out all the surgical instruments on an absorbent under pad in the order in which they will be used. Inspect the stereotactic frame to ensure that it is in proper operating condition. Ensure that all the manipulator arms move freely and smoothly with little to no sideways movement in the syringe holder. Direct the light beam at the ear bars and position the microscope such that the surgical area is visible through the eyepiece.
3. Place the mouse (female 6–8 weeks) under anesthesia by intraperitoneal (IP) injection of ketamine (75 mg/kg) and Dexmedetomidine (0.5 mg/kg). Before commencing surgery, administer Carprofen subcutaneously (5 mg/kg) as an analgesic and ensure that the animal is completely sedated by pinching the footpad or tail.
 - Rat Anesthesia dosage is as follows: Ketamine (75 mg/kg IP), Dexmedetomidine (0.25 mg/kg IP), and Carprofen (5 mg/kg SQ)
4. Using surgical shavers remove enough fur from the head of the animal to allow for aseptic procedures.
5. Carefully mount the animal onto the stereotactic frame and use the incisor bar to loosely immobilize the skull. Raise the animal to the level of the ears bars. Proceed by supporting the head of the animal and slide one of the ear bars into the ear canal and tighten in place. While keeping the head supported, slide the other ear bar into the proper position. The ears bars are meant to prevent any mediolateral head movement while maintaining the dorsoventral axis rotation free (see Note 2).
6. Use blunt forceps to open the mouth of the animal and insert the incisor bar. Press down gently to ensure the incisors are well seated. Swing the nose clamp over the nose of the animal and tighten gently. Ensure that there is no disruption in respiration. (see Notes 3 and 4).

3.2.2. Stereotactic Injection of Tumor Cells

7. Disinfect the incision area using alcohol wipes followed by of Tumor Cells Betadine. Apply ophthalmic lubricant to each of the eyes so they remain moist during surgery (see Note 4).

8. Using size 15 scalpels make a midline incision along the cranium roughly 1–2 cm in length beginning slightly posterior to the eyes and ending at the ears. Use the skin retractor to hold back the skin on both sides of the incision. The incision length for a rat is longer than a mouse as is the skin retractor.
9. Direct the light beams onto the exposed skull and focus the microscope on bregma; the junction of the sagittal and transverse sutures which should be visible under direct light. Use the blunt end of a scalpel and gently depress the parietal or frontal skull bone to assist in finding the junction (bregma) of the two bones (Fig. 4).
10. Position the syringe using the manipulator arms so that the needle tip is directly over bregma. Take the anteroposterior (AP) and mediolateral (ML) coordinates of bregma as these will serve as our starting coordinates.
11. Determine the coordinates of the injection site by adding or subtracting the appropriate lateral and anterior/posterior values. To inject into the striatum of a mouse move +0.5 mm AP then +2.2 mm ML from bregma. Coordinates for the rat striatum are +1.0 mm AP, +3.2 mm ML (see Note 5).
12. Visualize the injection site through eyepiece and use the drill to itch a small mark in the skull where the needle will penetrate. This mark is used as a guide for the operator to drill in the correct position. The needle can be lifted so that it does not obstruct the area.
13. With caution, drill a small burr hole with a 0.6-mm bit for mice and 1.75-mm bit for rats. This must be performed with extreme precaution as to avoid severing any blood vessels. Use a bent 28 G needle to ensure penetration of the dura mater. The rat skull is thicker and therefore more difficult to drill. For this purpose, use a 1.75-mm drill bit and drill in short bursts to avoid generating heat. Also, wet the area frequently with fresh saline solution, to avoid excessive heat (see Notes 8 and 9).
14. Load the Hamilton syringe (33 G needle for mice, 26 G for rats) with 1–5 μ l of cells of interest and expel a small amount onto a kimwipe to ensure that the syringe was properly loaded. Lower the needle such that it is level with the bur hole. Again read the dorsoventral coordinates and calculate the desired coordinate of the injection site. For injecting syngeneic mouse cells in the striatum slowly, move the needle down 3.5 mm and leave the needle in place for 1 min, then back up 0.3 mm to allow for a small pocket in which the cells can rest (–3.2 mm DV final). These coordinates will vary depending on the animal and cell line used. We have included the injection coordinates used by our laboratory to generate these glioma models in Figs. 1–3 and Notes 1 and 10.
15. Administer the injection slowly over the course of 3–5 min ($\sim$ 0.5–1 μ l/min). Keep the needle in place for an additional minute to allow tumor cells to settle before slowly withdrawing from the brain. Rat brains can accommodate a larger volume; therefore, we typically deliver a 3 μ l injection over the course of 3 min ($\sim$ 1 μ l/min) (see Notes 6 and 7).

16. To remove debris or tumor cells that might have escaped the tumor tract, flush the skull cavity with sterile saline and dry the area with a kimwipe.
17. Remove the skin retractor and close the incision using 3–0 nylon suture. If performing another injection, clean the syringe by flushing repeatedly with saline.
18. Resuscitate the mouse by IP injection of atipamazole (1 mg/kg IP). Once the mouse shows signs of voluntary movement, administer buprinex (0.05–0.1 mg/kg SQ) as an analgesic. Monitor the mouse until it fully recovers from anesthesia and return them to their original cage. A red light can be used as a heating source to aid in recovery as well as IP injection of warm ringer's solution. When working with nude mice, recovery is improved when performed in an infant incubator.
19. Provide the animals with water-soaked chow in a Petri dish and continue to monitor the animal for the following 7 days for any surgical complications. Remove any remaining sutures by day 14.

4. Notes

1. Maintaining the cell preparation on ice is very important for viability. The viability of the cell preparations decreases quickly over time; therefore, we recommend not using cell preps that have been on ice for more than 3 h. Reproducibility can be assessed by examining the time to mortality on Kaplan–Meyer survival curve (Figs. 1–3).
2. To generate reproducible tumor models, it is critical that the animal is immobilized properly in the stereotactic frame. Correct placement of the ear bars is challenging but is crucial for proper alignment of the skull. The ear bars must be inserted slowly and carefully into the ear canals of the animal without causing injury.
3. The operator should ensure that the dorsoventral plane of the cranium is as flat as possible. This can be adjusted by changing the vertical position of the incisor bar.
4. Mounting of the animal onto the frame should be performed with extreme care so that the position of the animal does not cause any respiratory issues. The breathing rate of the animal should be monitored during the entire procedure.
5. The stereotactic frame used is adequate for adult rats of 250 g body weight. Fitting larger animals into the frame may be difficult and the coordinates will also be inaccurate. The operator is advised to refer to a mouse or rat brain atlas for finding the coordinates of the injection site of interest (67).
6. The Hamilton syringes used to inject the cells must be clean and in good condition. The syringe must be clear of debris or blockage. The plunger should move smoothly up and down the barrel with little resistance. Hamilton syringe cleaner can be used to remove clogs. The syringe must be cleaned thoroughly with saline solution between each injection to avoid obstruction.

7. It is imperative that the injection bolus be administered over the course of several minutes to allow for the cells settle properly and not extravasate from the injection tract into the corpus callosum and external capsule.
8. The light source generates a significant amount of heat and should only be kept on when needed. The drill also generates a lot of heat. This is more of an issue for the rats since their skull is thicker and difficult to drill. It is recommended to drill in short bursts and not in a single step. Saline can be used to cool the area down before continuing. Any blood or CSF can be removed with cotton applicators or kimwipes. During the drilling step, the operator will have to refocus the microscope to clearly visualize the surgical area.
9. The brain is well vascularized and the operator is likely to encounter blood vessels during the drill step. We recommended drilling slowly and in the vicinity around the blood vessel to avoid rupturing the vessel directly. Doing so will slightly alter the coordinates of the injection.
10. Each of the cell lines grow in a distinctive pattern in vivo. This explains why some cell lines are injected at different coordinates. Typically, we create a pocket for the tumor cells to settle during the injection. To achieve the aforementioned pocket, we lower the needle 0.3 mm deeper than where we wish to inject. Then we lift the needle (0.3 mm) and administer the injection. For example, if injecting CNS-1 cells, the needle is lowered to -5.3 mm DV and then lifted to -5.0 mm where the cells will be administered. This step is typically done for all the tumor models and yields more consistent tumor profiles.
11. To prevent the growth of extracranial tumors, it is imperative to flush the skull with saline to remove any cells that might have traveled up the needle tract. Tumors should grow and remain encapsulated in the striatum as seen in Fig. 5.

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Fig. 1.

(a) Table with information relating to the biological features of syngeneic mouse GBM models including effective therapies. (b) Diagram depicting the stereotactic coordinates for injection of syngeneic mouse glioma cell lines as well as the cell number. (c) Kaplan–Meyer survival curve of mice bearing syngeneic intracranial brain tumors. C57BL/6 or VM/Dk mice implanted in the striatum with GL26, SMA560, B16-f10, or GL261 cells.

Fig. 2.

(a) Table listing the biological features of human xenograft GBM models including effective therapies. (b) Diagram depicting the stereotactic coordinates for injection of human glioma cell lines as well as the cell number. (c) Kaplan–Meyer survival curve of mice bearing syngeneic intracranial brain tumors. Rag1-deficient mice were injected in the striatum with U87, U251, and GBM12 cells.

Fig. 3.

(a) Table with information about the biological features of syngeneic rat GBM models including effective therapies. (b) Diagram depicting the stereotactic coordinates for injection of human glioma cell lines as well as the cell number. (c) Kaplan–Meyer survival curve of rats bearing intracranial tumors. Lewis or Fisher rats were injected in the striatum with CNS-1, F98, 9L, and RG2 cells.

Fig. 4. Schematic of a rat skull depicting the position of bregma relative to the frontal and parietal skull bones and the position for placing of the ear bars. Adapted from: *The Rat Brain in Stereotaxic Coordinates*, Academic Press, New York, 1998.

Fig. 5.

(a) Fisher rats were implanted in the striatum with 50,000 F98 cells or 500,000 9L cells, and Lewis rats were injected with 4,500 CNS-1 cells. Brains were harvested at the indicated time points. Microphotographs show the appearance of representative brain tumor sections stained with Nissl. (b) Nude mice were implanted with 1×10^6 U251 human glioma cells. Animals were sacrificed at the specified time points. Microphotographs show the appearance of representative brain tumor sections stained with Nissl.