A Syngeneic Mouse Glioma Model for Study of Glioblastoma Therapy

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Abstract. Animal models of human tumors serve a vital role in the development and testing of new anticancer therapies. Since the immune system is likely to play an essential role in tumor eradication, there is a particular need for modeling human disease in immunocompetent hosts. Few models of glioma have been developed in immunocompetent mice that are commercially available and none of these tumors have histological and antigenic characteristics of human gliomas. We have used a cell line, 4C8, derived from a spontaneous glioma-like tumor that arose in a transgenic mouse to develop a new glioma model. The intracranial injection of 4C8 cells into immunocompetent syngeneic B6D2F1 mice resulted in tumors that were densely cellular, developed a pseudopalisading pattern of necrosis, and expressed GFAP; all important features of human malignant gliomas. The average neurological endpoint was 51 days after intracranial injection. The 4C8 cells also grow rapidly in the flank, retaining histologic features seen in intracranial tumors. Flank tumors reached an average volume of 100 mm³, a volume ideal for therapy testing, by 34 days postinjection. These results suggest that the 4C8 mouse glioma model is an excellent system in which to test new antglioma therapies for use in humans.

Key Words: Brain tumor; c-neu oncogene; GFAP; Glioma, Syngeneic; Tumor model.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor in adults and, despite multimodal therapy, has a poor prognosis. Less than 5% of patients live beyond 3 years; a statistic that has not changed much over the past 2 decades despite improvements in adjuvant radiation and chemotherapy (1, 2). An essential component in the development of new therapeutic approaches for this disease is an animal model that closely mimics the human disease.

Currently, there are very few models of human GBM and those that are used most commonly do not accurately model the human disease. The ability of an animal model to predict the efficacy of new glioma therapies in humans is in part dependent upon the degree that the model system shares tumor histology and molecular marker expression with their human counterparts as well as sharing similar tumor behavior. Among the existing syngeneic transplantation animal gliomas, the rat 9L gliosarcoma cell line and mouse GL261 glioma tissue have been used most widely in recent years (3–12). The primary advantage of these models is that tumor growth is fatal uniformly within a time period reasonable for testing most therapeutic modalities. This permits reliable comparisons of treatment arms and control groups when evaluating new therapies.

Although many therapies have been tested in these systems, they do not accurately model human gliomas (4, 5, 7). When grafted intracranially, 9L tumors do not have histologic features of human gliomas; they grow as well-circumscribed masses that do not express glial markers such as glial fibrillary acidic protein (GFAP) (3, 5, 13). In addition, the 9L cell line originally was determined to be a gliosarcoma, but upon many culture passages, a sarcomatous cell component predominates (14). The most common mouse model of GBM, employing GL261 tissue, originally was described as an ependymoblastoma (5, 15), but has been described more recently as being poorly differentiated and lacking ependymal differentiation (3, 5). Like 9L gliosarcoma, GL261 grows in the brain as a well-circumscribed mass and also lacks expression of the important diagnostic glial molecular markers (15). The usefulness of the GL261 system also is compromised by the fact that the tumor cells do not exist as a stable cell line and must be maintained by subcutaneous serial transplantation, making in vitro studies difficult. Ideally, a GBM model would consist of glial-derived neoplastic cells that have a capacity for both in vitro growth, and intracranial and subcutaneous growth in syngeneic host animals. More importantly, derived tumors should have histological characteristics of gliomas and express glial markers. Recently, the CNS–1 rat glioma model has been described and appears to possess many of the above qualities (13). The CNS–1 model,
however, utilizes rats, which are useful for many studies but are more costly to procure and care for than mice, a significant disadvantage for extensive therapeutic studies. Our laboratory, therefore, became interested in generating a mouse glioma model that more accurately simulates human GBM.

To induce mouse tumors that have glial characteristics, 2 labs have used the strategy of expressing an oncogene under the control of a glial-specific promoter in transgenic animals. Danks et al. (16), generated transgenic mice that expressed the SV40 T antigen under the transcriptional control of the GFAP promoter (16). The tumors that spontaneously arose in these animals were not as aggressive as GBM, but were more consistent with low-grade astrocytomas. Cell cultures established from the astrocytomas could be injected into syngeneic mice with reliable tumor formation at the site of injection. The tumors grew very slowly, however, having a latency of 12–20 weeks after injection (16). Thus, although these cells were glial in character, the derived tumors did not have the histological characteristics of GBM and did not grow quickly enough to be a feasible model for testing of GBM therapies. In addition, this system utilizes the SV40 T antigen, a virally-derived gene not commonly expressed in human cancers. Features induced by SV40 T antigen expression, therefore, would be highly unlikely to enhance the human GBM-like nature of these tumors. A more accurate representation would employ an oncogene known to be altered in human GBM.

Hayes et al. (17) took such an approach when they expressed an activated neu oncogene (c-neu) under the transcriptional control of the myelin basic protein gene promoter (MBP) in transgenic mice (17). The investigators’ initial goal was to generate transformed oligodendrocytes, which influenced their decision to use the MBP promoter that normally is active in precursors of astrocytes and oligodendrocytes (17, 18). The neu oncogene encodes an integral membrane protein that is a member of the tyrosine kinase family of oncogenes, and originally was identified in cell lines derived from chemically-induced rat glioblastomas (19), demonstrating the potential of the oncogene to participate in the transformation of glial cells (20, 21). Transgenic MBP–c-neu mice spontaneously developed brain tumors that histologically and immunohistochemically were consistent with GBM, rather than the predicted oligodendrogliomas (17). Culture of cells from 1 of the tumors after dispersion generated a stable cell line, MOCH–1, that, depending on culture conditions, had astrocytic or oligodendrocytic properties (17). Later, Dyer et al. (18), derived and analyzed 15 MOCH–1 clones, ultimately isolating a homogeneous cell population, 4C8, that reliably expressed major myelin markers when grown in chemically-defined medium without serum, or GFAP when grown in 10% serum (18). The malignant glial features of the tumors that arose in the original transgenic animals, and the ability to propagate cells from the tumors in vitro, made them a very attractive possibility for generating malignant glial tumors in mice by transplantation. Thus, we have utilized the 4C8 cell line to establish flank and intracranial tumors in syngeneic B6D2F1 host mice to develop a new model of malignant glioma.

**MATERIALS AND METHODS**

**Cell Culture**

The 4C8 cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM) containing heat-inactivated 10% newborn calf serum (Life Technologies, Gaithersburg, Md.) plus 3.0 mg% glucose, 0.12% sodium bicarbonate, 50 μg/ml penicillin, 50 μg/ml streptomycin, 0.15 μg/ml Fungizone (Life Technologies), and 50 μg/ml metronidazole (Sigma, St. Louis, Mo.) at 37°C and 5% CO2. Cells were grown to confluence and split 1:2 every 4 to 5 days by detachment with 7.6% EDTA in phosphate-buffered saline (PBS). The 4C8 cells were tested for and confirmed to be free of mycoplasma by use of a MycoFluor mycoplasma detection kit (Molecular Probes, Eugene, Ore.).

**Animals and Tumor Implantation**

Six- to 8-week-old male mice of the B6D2F1 strain (Jackson Labs, Bar Harbor, Me.), the strain from which the transgenic mouse was generated, were used for all 4C8 studies. Animals were housed in American Association for Laboratory Animal Care-approved quarters and provided with unlimited access to food and water. Animals were quarantined for 7 days after arrival and prior to any procedures.

For intracranial and flank injections into B6D2F1 mice, 4C8 cells derived from passages 30 or 60 were cultured to confluence in medium containing 10% newborn calf serum for 7 days and then harvested by EDTA application. Cells were centrifuged and resuspended in fresh medium then mixed with an equal volume of Matrigel (Collaborative Research, Inc.). The final cell density was 1 × 10⁶ cells/5 μl for intracranial injections and 5 × 10⁷ cells/100 μl for flank injections.

For intracranial tumor growth studies, B6D2F1 mice were anesthetized with avertin (22) (50 mg/kg intraperitoneally) and then placed in a stereotactic head frame (David Kopf Instruments, Tujunga, Calif.). The scalp was opened and a small burr hole made 2 mm lateral to the bregma. The needle was inserted to a depth of 3.0 mm below the dura and 5 μl of cell suspension was injected over a period of 15 minutes (min). The needle was left in place for 1 min after injection and withdrawn slowly over 1 min. The burr hole was filled with bone wax and the scalp was closed with a clip. Mice were weighed every third day but observed daily for the development of signs of neurological distress. They were sacrificed if they were found to be moribund. This time point or, if the animal died between observations, the day of death, was defined as the neurologic endpoint.

To generate flank tumors, cells were injected into both flanks of each mouse and then the mice were observed daily for the initial appearance of the tumor nodule. Thereafter, the tumors were measured twice weekly and tumor volumes calculated for each animal using the following formula: (tumor length (mm)
× (tumor width)² (mm) / 2 (23). Growth ratios were calculated by measuring flank tumor volumes at sequential time points and comparing this to the initial volume of each tumor.

The 9L flank tumors were generated in adult female Fischer 344 rats (Harlan Sprague-Dawley) with 9L gliosarcoma cells obtained from Dr. Henry Brem (Johns Hopkins University) using the protocol described by Weiszsaecher et al (4). GL261 flank tumors were generated in 6- to 8-week-old female C3H/BL/6 (Jackson Laboratory, Bar Harbor, Me.) from a tumor fragment obtained from the NCI (Frederick, Md.) using the protocol described by Glick et al (8).

**Immunocytochemistry**

For immunofluorescence studies of cultured cells, 4C8 cells were grown on sterilized glass coverslips in the presence of 10% normal calf serum until semiconfluence was reached. The medium was removed and the coverslips were placed into either absolute ethanol at -20°C for 4 min or 4% paraformaldehyde for 10 min at room temperature. Coverslips then were processed as described in either Foley et al (24) or Dyer and Philibotte (18).

For immunofluorescence studies of tumors, whole brains with tumor transplants, or flank tumors were frozen in Tissue-Tek OCT embedding medium (Miles, Inc., Elkhart, Ind.), in a dry ice/ethanol slurry. Cryostat-generated 5 µm or 9 µm sections were then mounted onto Superfrost Plus slides (Fisher). The slides were immunolabeled as in Foley et al (25), and then mounted with coverslips as above.

For immunoperoxidase studies of tumors, 4% parafomaldehyde-fixed, paraffin-embedded 4 µm tissue sections were mounted on Plus slides. Following paraffin clearance and rehydration, the tumor sections and appropriate controls were immunolabeled using an indirect biotin-streptavidin method on a Ventana 320ES automated-immunomaster (Ventana Medical Systems, Tucson, Ariz.). For each of the monoclonal antibodies used, antigens were retrieved by microwaving in 0.01 M citrate buffer, pH 6, and for each of the polyclonal antibodies, sections were pretreated with trypsin prior to immunostaining. Biotinylated and horseradish peroxidase-conjugated secondary antibody reagents were purchased from Vector Laboratories (Burlingame, Calif.). Slides were counterstained using either hematoxylin or nuclear fast red.

The primary antibodies used and their sources were: the Z334 polyclonal antibody to GFAP at a dilution of 1:100 (DAKO, Carpinteria, Calif.); the A10 polyclonal antibody to synaptophysin at a dilution of 1:500 (DAKO); the polyclonal antibody to neuron specific enolase at a dilution of 1:300 (Zymed Labs, San Francisco, Calif.); the 3B4 monoclonal antibody to vimentin at a dilution of 1:100 (DAKO); the TUJ1 monoclonal antibody to type III β-tubulin, a gift from Dr. Anthony Frankfurter (Univ. of Virginia, Charlottesville); the AE1/AE3 monoclonal antibody to cytokeratin at a dilution of 1:100 (Boehringer Mannheim, Indianapolis, Ind.); the myelin basic protein polyclonal antibody (#664), a gift from Dr. David Colman, was used at a 1:2,500 dilution (26). Monoclonal antibodies to 2',3'-cyclic nucleotide 3'-phosphohydrolyase, galactocerebroside, myelin/oligodendrocyte specific protein, and myelin basic protein, used for 4C8 cell labeling only, were described previously (18). The secondary antibodies used for immunofluorescence were goat anti-rabbit-FITC antibody and goat antimouse-FITC antibody at a dilution of 1:50 (Kirkegaard and Perry Laboratories). Photomicrographs were generated on a Zeiss Axioshot using TMAX 400 film (Eastman/Kodak).

**RESULTS**

**Generation and Characterization of Intracranial Tumors**

The original description of the spontaneous tumors in MBPc-neu transgenic mice as glioblastoma multiforme-like (3, 17) and the subsequent ready growth of cell lines derived from those tumors (17, 18) made this system very attractive for development of an immunocompetent mouse model of glioma. One of the derived lines, 4C8, expressed astrocytic markers when grown in 10% serum, and expressed myelin proteins only when grown in chemically-defined medium without serum, as previously described (18). The cells grown in 10% serum for 7 or more days displayed a reactive astrocyte-like phenotype, i.e. star-shaped cells with numerous processes and concomitant GFAP expression, and thus, appeared to be good candidates for generating gliomas in vivo.

The 4C8 line grown in medium containing 10% serum for 7 days was tested for tumorigenicity in B6D2F1 mice. Following stereotactic injection of 4C8 cells into the right frontal cerebral hemisphere of 6 B6D2F1 mice, all animals were found to have tumors in the brain parenchyma that occupied the majority of the right frontal cerebral hemisphere (Fig. 1A). Intracranial tumor size increased over the time period examined (21–35 days) based on the sacrifice of 2 mice at each of 3 sequential time points (21, 28, and 35 days). Histologically, intracranial tumor masses were densely cellular and, although not diffusely infiltrating, were surrounded by nodules of tumor cells (Fig. 1B). The majority of the tumor cells contained pleomorphic nuclei with dense chromatin and occasionally, mitotic figures were observed (Fig. 1C). Scattered amongst the tumor cells were numerous giant cells containing intranuclear inclusions, irregularly lobulated nuclei and variable amounts of eosinophilic cytoplasm (arrow, Fig. 1C). Necrosis was not seen in the tumors from animals that were sacrificed prior to endpoint. No immune infiltrate was seen in any of the tumor specimens.

Three additional groups of animals were injected intracranially to determine whether the 4C8 tumors grew in a manner that would produce a reliable and reproducible neurological endpoint. All 38 animals in these studies experienced weight loss approximately 1 week prior to the onset of signs of neurological distress, such as hunched posture, weakness, or paralysis. Within 2 days of development of neurological signs, the mice became moribund or were found dead. This was defined as the neurological endpoint and was reached on average at 49 days postinjection in the initial group of 8 mice (Fig. 2).

The reproducibility of the tumor model was illustrated by
Fig. 1. Histology and immunohistochemistry of 4C8 brain tumors. A. A hematoxylin and eosin stained sagittal section showing a large 4C8 tumor in the brain of a mouse that reached neurologic endpoint 46 days postinjection. B. Nodules of tumor cells frequently are found at the margins of 4C8 brain tumors (arrows). C. High power magnification of intracranial tumor showing pleomorphic nuclei with dense chromatin and occasional mitotic figures. Giant cells are scattered amongst the tumor cells (arrow). D. Pseudopallidating necrosis that is characteristic of large brain tumors. E. In endpoint animals, tumors invade the ventricles (V) and the meninges (seen here in the interhemispheric fissure) (arrowhead). F. Expression of GFAP (brown) in 4C8 tumor cells. In A and E, bar = 1 mm. In B, C, D, and F, bar = 50 μm.

the analysis of 2 additional groups (each group n = 15) that received intracranial injections and reached average endpoints of 52 and 53 days postinjection (Fig. 2).

Gross examination of the brains from animals that reached endpoint indicated a swollen right cerebral hemisphere with occasional hemorrhagic staining apparent at the cortical surface. Sectioning the brain through the region of the presumptive intracranial tumor revealed a slightly gray mass that contained central regions of necrosis. Histologic sections indicated that the swollen appearance of the brain likely was due to both the large size of the tumor and edema of the surrounding brain...
tissue. There was significant compression of brain structures secondary to the large size of the tumors. The sections also confirmed that the tumor had areas of necrosis, as suggested by their gross appearance, and, significantly, this necrosis had a pseudopallisading appearance as commonly seen in human GBM’s (Fig. 1D). Finally, 25% of the examined endpoint animals had tumor throughout their ventricular systems with ventricular enlargement and occasional small nests of tumor in the leptomeninges (Fig. 1E). No extracranial growth was observed in any of the 48 total animals intracranially injected in these studies.

Since the 4C8 cell-derived tumors displayed histologic characteristics of malignant glial neoplasms, an immunohistochemical study was performed to determine whether astrocytic markers were expressed. For this and subsequent labeling studies described below, 9L and GL261 tumors grown in the flank were sectioned, labeled, and compared to intracranial 4C8 tumors. Tumors of 4C8 cells had a patchy distribution of GFAP expression that was found in the cytoplasmic processes of approximately 10–20% of the tumor cells (Fig. 1F). GFAP-positive cells that had the appearance of host-derived reactive-astrocytes also were found within the tumor mass, but could be distinguished easily from tumor cells by virtue of their distinctive morphologies. The 9L gliosarcoma and GL261 flank tumors did not contain any GFAP-antibody-reactive protein. Since 4C8 cells were shown to express myelin markers in a chemically-defined medium without serum, we tested whether the tumors,

Fig. 2. Survival curve for B6D2F1 mice receiving intracranial injections of 4C8 tumor cells indicated that 4C8 intracranial injection leads to reproducible endpoint by 51 days (average of the 3 10% serum studies) and is uniformly fatal by 64 days postinjection. Study 1 and 2 were completed with cells from passage #30 (n = 15 and 15, respectively), study 3 was completed with cells from passage 60 (n = 8).

Fig. 3. Average growth ratios of 4C8 xenografts established in the flanks of B6D2F1 mice are plotted ± SEM. An average tumor volume of 100 mm³ was reached by 34 days postinjection (10% culture conditions). For both studies 5 animals were injected for a total of 10 flank tumors for each culture condition.

even though derived from cells prepared under very different conditions, expressed a myelin marker. The 4C8-derived tumors, in contrast to white matter in the same brain sections, were completely unlabeled by the polyclonal antibody to myelin basic protein. The 4C8 and GL261 tumors did not express the epidermal growth factor receptor, keratin, or the primitive cell maker vimentin, all found commonly in sarcomas, whereas 9L gliosarcoma was positive for these markers. This supports the previous characterization of 9L as a sarcoma. None of the tumors expressed the neuron-specific markers type III β tubulin, synaptophysin, or neuron specific enolase.

Generation and Characterization of Flank Tumors

Evaluation of potential therapies is more convenient when tumors can be serially measured, such as those established subcutaneously in the flanks. To determine the characteristics and growth rates of 4C8 flank tumors, 5 B6D2F1 mice received bilateral subcutaneous flank injections of 4C8 cells grown in medium containing 10% serum. At 14 days postinjection, small flank tumors could be palpated at 5/10 (50%) of the injection sites and by 18 days postinjection, tumors were palpated at 10/10 (100%) of the injection sites. Beginning at day 14 postinjection, growth ratios were calculated for each of the 10 tumors and then averaged and plotted over time (Fig. 3). A linear phase of tumor growth rate was seen until the average tumor volume reached approximately 10 mm³, corresponding to day 28 postinjection. After day 28, the flank tumors grew logarithmically reaching an average tumor volume of 100 mm³ by 34 days postinjection.

Tumor growth was assessed until day 39 when the tumor burden affected the health of the mice, necessitating
euthanasia. Gross examination indicated that the tumors were firm, cohesive masses that appeared to be sharply circumscribed from surrounding tissues. Section of each tumor with a scalpel allowed observation of areas of central necrosis present in every tumor examined. Histological studies showed that the flank tumors were indistinguishable from the intracranial masses, except that the flank tumors had larger areas of necrosis (data not shown). Flank tumor sections also were immunolabeled in a manner similar to the intracranial tumor sections and their tumor antigen profiles proved to be consistent with that of the intracranial tumors (data not shown).

**DISCUSSION**

Intracranial and flank tumors derived from the 4C8 cell line possess many properties of human gliomas and meet many of the criteria of an "ideal" glioma model (3, 7). Importantly, 4C8 tumors are generated from glial-derived neoplastic cells that are propagated readily in vitro. The induction of the astrocytic phenotype upon culture in 10% serum has been consistent throughout the growth of these cells in Philadelphia (18) and at the University of Cincinnati. The robust in vitro growth of these cells provides a reliable, renewable source of tumor material. The 4C8 cell line is propagated at either intracranial or subcutaneous sites generated tumors reproducibly at all injection sites. The cell line appears to be extremely stable as injection of cells from passage 60 (study 3) produced tumors on a similar time scale and that were histologically indistinguishable from tumors generated from passage 30 (Fig. 2, studies 1 and 2). Intracranial growth of 4C8 cells generally was circumscribed, although satellite nodules of tumor cells that were reminiscent of human glioma growth consistently were observed. Similar to human GBM, the 4C8 tumors often extended to the ventricular system. Two of the 8 animals analyzed had tumor that extended into the lateral ventricles, a feature occasionally seen in humans with GBM.

Tumors derived from injection of 4C8 cells model the histology and tumor antigen expression seen in human malignant gliomas (2, 27). Like human glioblastomas, these tumors are densely cellular, have satellite nodules at tumor borders, and develop a pseudopalisading pattern of necrosis. Tumor cells also express GFAP, a diagnostic marker of human gliomas. This marker is expressed variably in human malignant gliomas, depending on the level of astrocytic differentiation (28, 29). Like less differentiated, highly malignant human GBM (28, 29), approximately 10–20% of 4C8 tumor cells expressed GFAP in 4C8 tumors. This is similar to the proportion of high GFAP-expressing 4C8 cells in vitro. It is important to note that the GBM-like histologic and antigenic profile of 4C8 tumors does not require the context of the central nervous system for its development as demonstrated in the flank tumor experiments.

Finally, and perhaps most important to future studies of glioma therapy, intracranial injection of 4C8 cells is fatal uniformly within a time period that is reasonable for testing most therapies, including those dependent on a host immune response. Mice examined at endpoint have a large tumor burden that probably causes death of the animal by compressing critical brain structures as would occur in untreated humans with GBM. Since immunemediated mechanisms of tumor killing appear to be critical to most, if not all modes of cancer gene therapy, the development of the 4C8 model in immunocompetent, commercially available B6D2F1 mice is a significant addition to existing models for the testing of glioblastoma therapies.

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