As a result of recent advances in surgical techniques, radiation therapy, and chemotherapy, management of malignant gliomas has improved. Unfortunately, the prognosis for glioblastoma multiforme and anaplastic astrocytoma has seen little improvement and remains poor, with patients facing a median survival time of 9 months and only 5 to 10% of patients surviving to 2 years. Most of these patients die of local tumor recurrence or regrowth, which in most cases is difficult to treat. In general, a recurrent glioma features drug resistance and a more aggressive invasive phenotype. Although gliomas have a tendency to increase in malignancy during their natural course, recurrent gliomas may increase in malignancy compared with the original tumor, possibly in relation to treatment.

Tumors consist of discrete cell subpopulations that are heterogeneous with respect to karyotype, DNA content, morphological characteristics, growth rate, and drug response. The effect of treatment on processes generating phenotypical heterogeneity and the development of resistance is poorly understood for the majority of treatment modalities. Chemotherapy may result in the selection or induction of cells with altered sensitivity to the treatment; in some cases, a population selected for resistance to one agent may be resistant to other agents as well.

In this regard, a recurrent glioma or a glioma remaining after conventional treatment may have very different characteristics from the original tumor. Our recent findings focused on different characteristics of invasiveness found in drug-resistant sublines of 9L rat glioma cells. Although gliomas have a tendency to increase in malignancy during their natural course, recurrent gliomas may increase in malignancy compared with the original tumor, possibly in relation to treatment.
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spheroids in culture after 1 week of BCNU exposure in vitro. Both cell lines displayed increased invasiveness compared with 9L cells when implanted into the striatum of Fisher 344 rats. Because BCNU is the most common chemotherapeutic agent used in glioma therapy, it is important to study these cell lines, which seem to mimic the characteristics of a drug-resistant recurrent glioma. Recently many newly developed treatment strategies that seemed promising in animal experiments have exhibited poor results when introduced into the clinical setting. Animal models used to develop these new treatments are considered to be partial causes of these discrepancies. The tumor model U87MG, which is often used in athymic rats, grows into a well-circumscribed mass, as does the 9L gliosarcoma. In addition the 9L sarcoma shows sensitivity to BCNU treatment. Therefore, there is a need for an invasive drug-resistant brain tumor model that can be easily established and used. Hypothesizing that a tumor model based on the 9L-2 cell line could satisfy these requirements, we tried to establish a 9L-2 glioma brain tumor model.

Materials and Methods

Tumor Lines

Three established rat glioma cell lines (9L, 9L-2, and BTRC-19) were used in this study. The 9L-2 and BTRC-19 cell lines were obtained from the Brain Tumor Research Center Tissue Bank at UCSF and the 9L cell line was obtained from the Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, Bethesda, MD (Stuart Walbgidge, B.S.). The cells were seeded into tissue culture flasks 2 or 3 days before implantation into a rat brain and were grown as monolayers in CMEM consisting of Eagle MEM supplemented with 10% fetal calf serum and nonessential amino acids. The cells were cultured in an incubator at 37˚C in a humidified atmosphere composed of 95% air/5% CO2. Cells were harvested by trypsinization, washed once with CMEM, and resuspended in PBS for implantation.

In Vitro Effect of BCNU on Tumor Cells

All 9L, 9L-2, and BTRC-19 cells were seeded separately into 24-well cell-culture dishes at a concentration of 5 × 10⁴ cells per well. After a 24-hour incubation, the medium was replaced by media containing BCNU at concentrations of 50, 100, and 200 μM. The BCNU was a generous gift from Dr. William Bodell at UCSF. A stock solution of BCNU was prepared by reconstituting the BCNU in PBS containing 10% ethanol. When using stock for cells, the stock solution was diluted at least 100-fold in the medium. After 48 hours of incubation, the cells were harvested by trypsinization, mixed with trypan blue dye, and counted using a hemacytometer.

Animal Preparation

Male Fisher 344 rats (Harlan, Indianapolis, IN), each weighing between 200 and 250 g, were housed in standard facilities and given free access to water and food. All protocols used in the animal studies were approved by the UCSF Committee on Animal Research.

Intracranial Tumor Implantation

Nine rats were divided into three groups (three animals per group), and underwent implantation of 9L, 9L-2, or BTRC-19 cells, depending on the group to which they were assigned. The animals were anesthetized with ketamine (10 mg/kg) and xylazine (3 mg/kg), which were administered by an intraperitoneal injection. Afterward they were placed in a small-animal stereotactic frame (Kopf, Tujunga, CA). A sagittal incision was made through the skin and a burr hole was created in the skull by using a twist drill. The cannula coordinates were 0.5 mm anterior to the bregma and 3 mm lateral from the midline. A cell suspension (5 × 10⁴ cells/10 μl) was used for tumor implantation. Five microliters of this suspension was injected at the depth of 4.5 mm from the brain surface. Following a 2-minute waiting period, another 5 μl was injected at the depth of 4 mm. After another 2-minute waiting period, the needle was removed and the wound was closed using a nylon suture.

Brain Tissue Staining

Ten days after tumor implantation, the animals were killed and perfused with 10% formalin. Their brains were removed and placed in 10% formalin for another 24 hours. After fixation, the brains were processed to provide frozen sections. The frozen sections, 40-μm thick, were cut using a cryostat and then stained with H & E.

Staining for Proliferating Cells in the 9L-2 Brain Tumor Model

An additional three rats received implants of 9L-2 cells and were killed 10 days after implantation. Their brains were processed to provide paraffin-embedded sections and 5-μm-thick sections were cut using a microtome. Immunohistochemical staining was performed by using the Ki-67 antibody (1:200 dilution; BD Bioscience, San Jose, CA). Briefly, the slides were deparaffinized with xylene and rehydrated with descending concentrations of ethanol (100–70%). Antigen retrieval was performed by soaking the slide in sodium citrate buffer (pH 6.1) and heating it in a microwave oven. After antigen retrieval, the slides were incubated with 0.9% hydrogen peroxide and blocking solution (1% normal goat serum and 0.01% Triton X-100 in PBS) to reduce nonspecific background staining. The primary antibody was applied overnight at room temperature. On the following day, biotinylated anti–mouse serum (1:300 dilution in blocking solution; Vector Laboratories, Inc., Burlingame, CA) and streptavidin–horseradish peroxidase (1:300 dilution in PBS; Vector Laboratories, Inc.) were applied. A 3,3-diaminobenzidine staining kit (Vector Laboratories, Inc.) was used to make the bound antibody visible.

Western Blot Analysis for Phosphorylated Akt, Akt, and Actin

 Cultured cells that had reached 70% confluence were used for this study. The cells were washed with PBS and cultured in serum-free medium for 6 hours. After this step the cells were collected and protein was extracted using cell lysis buffer (Cell Signaling Technology, Inc., Beverly, MA). Equal amounts of protein were separated using a
7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto a polyvinylidine difluoride membrane (BioRad Laboratories, Hercules, CA). The polyvinylidene difluoride membrane was incubated in blocking buffer (Tris-buffered saline–casein blocker; BioRad Laboratories) and afterward with an anti–phosphorylated-Akt antibody (Phospho-Act [Ser 473] antibody; Cell Signaling Technology, Inc.) (1:500 dilution at 4˚C overnight), anti-Akt antibody (Cell Signaling Technology, Inc.) (1:500 dilution at room temperature for 1 hour), and anti–β/Actin antibody (Sigma Bioscience, St. Louis, MO) (1:1000 dilution at room temperature for 1 hour). The blot was made visible by using a colorimetric detection kit (Opti-4CN detection kit; BioRad Laboratories).

Measurement of MMP Activity

The cells were seeded into 6-well cell-culture plates (50 × 10^4 cells/well) and incubated in CMEM for 24 hours. The cells were then washed twice with serum-free MEM and incubated in CMEM for another 48 hours. The medium was collected and centrifuged to remove cellular debris. The supernatant was mixed with a zymogram sample buffer (all Zymogram products purchased from BioRad Laboratories). The activities of gelatinases (MMP-2 and MMP-9) were analyzed using the gelatin zymography technique. Twenty or 45 microliters of samples were loaded onto the zymogram gel. The gel was electrophoresed at 200 V, after which it was incubated in a Zymogram renaturing buffer (Triton X-100, 25% [vol/vol] in water) for 30 minutes at room temperature. The Zymogram renaturing buffer was replaced with Zymogram developing buffer and incubated for 24 hours at 37˚C. Finally, the gel was stained with 0.5% (wt/vol) Coomassie Blue R-250 for 30 minutes and excess stain was removed using glacial arctic acid.

Evaluation of Tumor Size and the Survival Study: 9L-2 Brain Tumor Model

Seventeen male Fisher 344 rats were used to establish the 9L-2 brain tumor model. The cells were implanted in the rats in the manner described for the staining study. On 7, 10, 14, and 21 days after tumor implantation, three rats per day were killed and their tumor sizes were evaluated. Tumor volumes were calculated according to the formula

\[ v = \frac{1}{6} l \times w^2 \]  

or the formula

\[ v = \frac{1}{6} l \times w \times d \times \pi^6 \],

in which l is the greatest length, w is the width of the perpendicular axis, and d is the depth. Five rats were observed to determine the duration of survival after tumor implantation.

Results

In Vitro Effect of BCNU

Three different concentrations of BCNU were tested for each cell line. Compared with 9L cells, which are sensitive to BCNU treatment, 9L-2 and BTRC-19 cells showed an increased resistance to BCNU treatment (Fig. 1).

Invasive Phenotype Observed in BCNU-Resistant Sublines in Vivo

The growth patterns of tumors grown in the brains of Fisher 344 rats were compared using H & E staining. Whereas the original 9L cells developed into a well-circumscribed mass in the brain, the 9L-2 and BTRC-19 cells each developed into a tumor mass with invasive margins extending into the surrounding normal brain (Fig. 2).
The 9L-2 cells were more invasive than the BTRC-19 cells, but the invasive phenotype observed in both of these cell lines was different from the original 9L cells. Using anti–Ki-67 staining, which stains proliferating cells, we also found positively stained cells scattered in regions distant from the main tumor mass in the animals with 9L-2 gliomas (Fig. 3). Because the 9L-2 cells developed into more invasive tumor masses than the BTRC-19 cells, the former cell line was used for further characterization as a brain tumor model.

**Phosphorylation of Akt and Gelatinase Activity**

Phosphorylation of Akt at Ser473 was evaluated by performing a Western blot analysis. An apparent enhancement in phosphorylation of Akt was found in 9L-2 cells compared with 9L cells.

Phosphorylation of Akt was also slightly elevated in the BTRC-19 cells when compared with that in the 9L cells (Fig. 4A). Gelatin zymography was used to determine gelatinase activity in the cell culture medium. This test revealed enhanced activity of both MMP-2 and MMP-9 in the 9L-2 and BTRC-19 cells, albeit more so in the 9L-2 cells, when compared with the 9L cells (Fig. 4B). This finding strongly indicates a change in those cells to more invasive phenotypes.

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Growth of 9L-2 cells in the brain was relatively consistent among the animals as demonstrated in Fig. 5. The incidence of tumor was 100%, with a survival period after cell implantation of 22.4 ± 4.9 days (observed in five animals).

**Discussion**

The chemotherapeutic agent BCNU is one of the most commonly used against malignant brain tumors; its effectiveness has been demonstrated in basic and clinical studies. Nevertheless, most patients experience local recurrence or regrowth of their tumors following BCNU treatment. Moreover, tumors that survive the initial treatment are usually much more difficult to control. Although diverse processes govern therapeutic responses to a drug, it seems that BCNU chemotherapy may result in the selection or induction of cells with an altered sensitivity to the drug. Our findings in 9L-2 cells and BTRC-19 cells,
which are BCNU-resistant sublines of the 9L gliosarcoma line, demonstrate the increased invasiveness of these drug-resistant cells. The precise mechanisms for this phenotypical change are unclear; however, the fact that these two cell lines developed differently from the 9L line after in vivo or in vitro exposures to BCNU indicates the positive role of chemotherapy in the selection or induction of more invasive tumors.

Recently, a dichotomy of migration and proliferation has been reported. In a cell population, migration and proliferation are antagonistic cell behaviors. If an invasive glioma displays a predominantly migratory cellular phenotype with a temporarily lowered proliferation rate, these cells may be relatively resistant to conventional cytotoxic treatments, which are frequently directed against proliferating cells. Moreover, there is increasing evidence that invasive glioma cells with a low propensity for proliferation may also be resistant to apoptosis. Mariani and associates demonstrated that glioma cell motility is associated with a reduced transcription of proapoptotic and proliferation genes. Once treated with chemotherapeutic agents, subpopulations of tumors that display increased motility with less expression of proapoptotic genes may survive. The 9L-2 and BTRC19 cell lines may have been developed from these subpopulations of the original 9L tumor. From the molecular point of view, in our study both 9L-2 and BTRC-19 cells revealed higher levels of phosphorylated Akt, which indicates increased phosphatidylinositol 3-kinase signaling. This increase in phosphatidylinositol 3-kinase signaling has been attributed to increased invasiveness and gelatinase activity in malignant gliomas. We speculate that this may be one cause of the morphological changes acquired by these BCNU-resistant sublines. An elevated activity of O'-alkylguanine DNA alkyltransferase, which accounts for BCNU, resistance has already been reported in 9L-2 and BTRC-19 cells. In these sublines we also found increased expression of P-glycoprotein, a product of a multidrug-resistant gene (data not shown). All these findings together seem to indicate increased malignancy in the 9L-2 and BTRC-19 sublines.

In this study we established the 9L-2 brain tumor model, Most murine and human xenotransplant models of gliomas are somewhat noninvasive, and attempts to identify invasive tumor models have been undertaken in the past. With the high incidence of tumor uptake (100%), and the relatively consistent growth, and invasive nature of the tumors, this model will be valuable in the examination of drug-resistant invasive malignant gliomas.

Our findings have implications for the chemotherapy of gliomas. If treated with BCNU in an insufficient manner, tumors may develop a resistance to BCNU, or cells already relatively resistant to BCNU may be selected and demonstrate a more aggressive migratory phenotype. In fact, this is the case with many recurrent gliomas, which are hard to control and are lethal. Thus, the development of new therapeutic strategies that can target drug-resistant and invading tumor cells needs to be explored. The 9L-2 brain tumor model established in this study, which appears to mimic drug-resistant recurrent malignant gliomas, will aid in the future development of treatment strategies against drug-resistant invasive malignant gliomas.

Conclusions

In this study we demonstrated phenotypical changes in the 9L-2 and BTRC-19 sublines (derived from the 9L gliosarcoma cell line) that were characterized by a resistance to BCNU. Both cell sublines exhibited an increase in invasive properties when implanted into the central nervous system of Fisher 344 rats compared with the original 9L gliosarcoma. Because the 9L-2 and BTRC-19 cells were derived from 9L cells treated with BCNU (in vivo or in vitro) the change in phenotype was likely caused by the drug treatment, which may have clinical implications for
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the chemotherapeutic treatment of gliomas. In addition, the rodent tumor model that was created using the 9L-2 cells may serve as an effective drug-resistant and invasive brain tumor model that can be used to develop future treatment strategies.

Acknowledgments

We thank William Bodell, Ph.D., Dennis Deen, Ph.D., and Tomoko Ozawa, M.D., Ph.D., of the Department of Neurological Surgery, UCSF, for their kind advice, and Mrs. Sharon Reynolds of the Department of Neurological Surgery, UCSF, for editorial help.

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Manuscript received November 6, 2003. Accepted in final form June 28, 2004. This study was funded in part by grants from National Brain Tumor Foundation (K.S.B.) and Accelerated Brain Cancer Cure (K.S.B.).

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J. Neurosurg. / Volume 101 / November, 2004
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