Isolation and characterization of human malignant glioma cells from histologically normal brain

DANIEL L. SILBERGELD, M.D., AND MICHAEL R. CHICOINE, M.D.

Department of Neurological Surgery, Washington University School of Medicine, St. Louis, Missouri

Malignant gliomas pose a formidable therapeutic challenge because of their invasive growth characteristics. Glioma cells that have infiltrated beyond gross tumor into histologically normal brain cannot be completely removed surgically; they are protected by an intact blood-brain barrier and do not appear to enter mitosis, thus making radiotherapy and chemotherapy less effective. Malignant glioma cells invade beyond abnormalities detected with computerized tomography and magnetic resonance (MR) imaging; they often invade the brain more than 2 cm beyond the gross tumor, are not usually found within the confines of a single carotid artery or vertebral artery distribution, and are shown at autopsy to have extended farther than predicted antemortem. However, in previous studies determination of the extent of tumor cell invasion relied on the identification of tumor cells in surgical or autopsy specimens using various standard staining techniques. Until now efforts have not been made to initiate tumor cells from histologically normal brain in culture to document their presence and to characterize these important cells biologically.

Brain invasion prevents complete surgical extirpation of malignant gliomas; however, invasive cells from distant, histologically normal brain previously have not been isolated, cultured, and characterized. To evaluate invasive human malignant glioma cells, the authors established cultures from gross tumor and histologically normal brain. Three men and one woman, with a mean age of 67 years, underwent two frontal and two temporal lobectomies for tumors, which yielded specimens of both gross tumor and histologically normal brain. Each specimen was acquired a minimum of 4 cm from the gross tumor. The specimens were split: a portion was sent for neuropathological evaluation (three glioblastomas multiforme and one oligodendroglioma) and a portion was used to establish cell lines. Morphologically, the specimens of gross tumor and histologically normal brain were identical in three of the four cell culture pairs. Histochemical staining characteristics were consistent both within each pair and when compared with the specimens sent for neuropathological evaluation. Cultures demonstrated anchorage-independent growth in soft agarose and neoplastic karyotypes. Growth rates in culture were greater for histologically normal brain than for gross tumor in three of the four culture pairs. Although the observed increases in growth rates of histologically normal brain cultures do not correlate with in vivo behavior, these findings corroborate the previously reported stem cell potential of invasive glioma cells. Using the radial dish assay, no significant differences in motility between cultures of gross tumor and histologically normal brain were found.

In summary, tumor cells were cultured from histologically normal brain acquired from a distance greater than 4 cm from the gross tumor, indicating the relative insensitivity of standard histopathological identification of invasive glioma cells (and hence the inadequacy of frozen-section evaluation of resection margins). Cell lines derived from gross tumor and histologically normal brain were usually histologically identical and demonstrated equivalent motility, but had different growth rates.

KEY WORDS • astrocytoma • brain neoplasm • cell culture • cell motility • glioblastoma multiforme • oligodendroglioma

The motility of normal glia that is observed during embryogenesis and following cerebral injury is retained and amplified in malignant glioma cells. In vivo studies in rats have demonstrated that prelabeled implanted C6 glioma cells quickly disperse along white matter tracts, thus allowing tumor cells to be identified in the contralateral hemisphere within 2 weeks. Gross tumor forms at the site of implantation, whereas invading cells remain single. In vitro studies using the radial assay technique indicate that human malignant glioma cells move from regions of high cell density to regions of low cell density. This movement is dependent on serum concentration (increasing with higher serum concentrations), is reliant on cytoskeletal assembly (blocked by cytochalasin B and enhanced by exposure to taxol), and varies with the type of tumor and the degree of histologically determined malignancy (tumor grade). The addition of hydroxyurea to arrest cells in the gap phase (G₀) does not alter the observed in vitro motility. Time-lapse videomicroscopy demonstrates ruffling of the leading edge of the cell, the development of typical pseudopodia, and a mean linear velocity of approximately 12.5 μm/hour.
Malignant gliomas tend to recur at the margins of surgical resection regardless of the extent of the resection or the distance that the resection extends from the gross tumor. Even when surgical hemispherectomy is undertaken, the tumor recurs at the resection margin. One possible explanation is that because tumor cell density is greatest at the resection margin, there is a higher probability of recurrence. Another possibility is that a minimum tumor cell density must be achieved to facilitate tumor growth into a bulky state. However, neither of these hypotheses explains why distant tumor cells do not commonly form clusters of cells, leading to secondary bulk tumors or gliomatosis cerebri. Following implantation in rats, C6 rat glioma cells that have invaded the contralateral hemisphere can be reestablished in culture; when implanted into naive rats, these cells will form gross tumors, thus confirming the retained stem cell capabilities of these invading cells.

A major void in malignant glioma research has been created by the fact that investigators have focused primarily on cells contained in the gross tumor. Establishing cultures of human glioma cells from histologically normal brain would permit biological characterization of these important cells. Therefore, the specific aims of this study were constructed to begin the process of elucidating biological characteristics unique to invasive human glioma cells. To accomplish this, we initiated in culture cell lines derived from paired surgical specimens obtained from gross tumor and histologically normal brain. Specimens of histologically normal brain were obtained from areas more than 4 cm from the edge of the gross tumor (as defined by MR imaging and by intraoperative gross inspection and ultrasonography). Each surgical specimen and cell line was characterized by histochemical staining, growth rate in culture, anchorage-independent growth in soft agarose, and in vitro motility.

Materials and Methods

Acquisition of Human Glioma Specimens

Surgical specimens, obtained during routine craniotomies for resection of malignant supratentorial gliomas in adults, were classified either as gross tumor or as histologically normal brain (that is, brain located more than 4 cm from the gross tumor, as measured at the time of resection using both gross landmarks and intraoperative ultrasonography and corroborated postoperatively with contrast-enhanced MR imaging). The extent of the resection and standard neurosurgical craniotomy methods were not altered in any way for this investigation. All clinical procedural plans were reviewed and approved by the Washington University internal review board on human subjects. Four surgical procedures (two frontal lobectomies and two temporal lobectomies), which were performed over a 3-year period, yielded specimens of both gross tumor and histologically normal brain. These operations were performed in three men and one woman with a mean age of 67 years.

The specimens were split into two portions: one part was used for culture and the other for routine neuropathological evaluation, including hematoxylin and eosin staining and immunocytochemical staining for glial fibrillary acidic protein (GFAP). Bulk tumor specimens were graded according to World Health Organization criteria.

Establishment of Malignant Glioma Cell Lines

The techniques for culturing and maintaining cell lines of malignant gliomas have been previously reported. These techniques were used for specimens of both gross tumor and histologically normal brain. Briefly, fresh surgical specimens, transported in sterile lactated Ringer’s solution, were minced to a slurry with crossed scalpels, centrifuged at 250 G for 10 minutes at 4°C, and resuspended in calcium- and magnesium-free Hank’s Balanced Salt Solution (HBSS) containing a triple enzyme mixture of 0.04% collagenase, 0.02% deoxyribonuclease, and 0.2% neutral protease. The cell suspension was incubated at 37°C for 60 minutes, washed in Dulbecco’s modified Eagle’s medium (DMEM) via centrifugation at 250 G for 12 minutes at 4°C, and resuspended in DMEM with 20% heat-inactivated fetal calf serum (FCS), 0.2 mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml neomycin. Cell lines were expanded and then stored in a dimethyl sulfoxide solution in liquid nitrogen for later use (viability after thawing and rephasing is typically 93–98%). To minimize the influences that can arise from growing cells in culture, low-passage cells (>3 passages, <10 passages) were used for all the studies described in this report.

Immunocytochemical Staining for GFAP

To determine the expression of GFAP, an intermediate filament present only in benign and neoplastic astrocytes, a commercially available GFAP immunoperoxidase staining kit was used. Briefly, 2 × 10⁶ cells were plated in chamber slides for 24 hours at 37°C in a humidified 5% CO₂ atmosphere and then fixed in 95% alcohol for 1 hour at room temperature. The slides were placed in a diluted blocker for 5 minutes to neutralize the endogenous peroxidase activity and then they were incubated at room temperature as follows: 5 minutes with tissue conditioner, 30 minutes with primary antibody, 30 minutes with secondary antibody, 30 minutes with peroxidase reagent, and 15 minutes with the chromogen reagent. Between incubations, the slides were rinsed for 3 minutes with buffer. Hematoxylin was used as a counterstain. The slides were rinsed in Scott’s Tap Water, mounted, and viewed by microscopy. An established rat astrocytoma cell line, C6, with a high level of GFAP positivity, was used as a positive GFAP control.

Growth Rate Determinations

The growth rates of tumor cell lines were determined over a 14-day period as previously described. Briefly, cells were plated at 10⁴ cells/well in six-well, flat-bottom culture plates. After 24 hours, the plating medium was aspirated and 3 ml of feeding medium was added to each well. At 24-hour intervals, three wells were washed with calcium- and magnesium-free HBSS. To remove the cells from the wells for counting, the cells were exposed to 1 ml of trypsin–ethylene diamine tetraacetic acid (EDTA) (315 U/ml of trypsin activity and 0.2 M EDTA) for 3 minutes. Trypsination was halted by addition of 4 ml of feeding medium. The cells were centrifuged at 250 G for 12 minutes at 4°C, and resuspended in 1 ml of medium, after which they were counted using a hemocytometer. The cell counts were made in duplicate for each of these wells (six counts each day). Growth rate was defined as the mean percentage increase in cell number per day.

Anchorage-Independent Growth in Soft Agarose

Anchorage-independent growth in soft agarose was used to verify that the cultured cells were neoplastic. Cells near confluency (midlogarithmic growth phase) in T-75 flasks were washed with calcium- and magnesium-free HBSS and exposed to 3 ml of trypsin solution (315 U/ml of trypsin activity and 0.2 M EDTA) for 60 seconds at 37°C. The cells were then resuspended in 7 ml of DMEM with 10% FCS, centrifuged at 250 G for 5 minutes at 4°C, and resuspended in DMEM (2 U/ml insulin, 0.4 mM 1-glutamine, 0.4 mM 1-asparagine, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml neomycin). To each 0.9 ml of cell suspension, 0.1 ml of 2.5% agarose (sea plaque agarose, melted and then cooled to 37°C in a water bath) was added and then loaded into capillary tubes prepared using 10-μl borosilicate micropipettes cut to a length of 9.75 cm. Following capillary tube loading, the ends of the tubes were occluded with hematocrit clay, placed in labeled borosilicate tubes in a rack at a 45° angle, and incubated at 37°C. On Day 14, the capillary tubes were taken from the incubator and their ends were removed.
with a glass cutter. A piece of rubber tubing and a mouthpiece were attached to a micropipette tip, which was then attached to the micropipette, and the agarose–cell suspension was gently blown onto a microscope slide and examined using light microscopy to identify colony formation (> 50 cells and > 50 μm).

Chromosomal Analysis

To verify that all cell cultures were composed of cells with neoplastic karyotypes, chromosomal analysis was performed by a commercial laboratory blinded to the type of cells being tested. The laboratory was asked to determine whether the karyotypes delineated were consistent with neoplastic or normal cells. Briefly, cells frozen in dimethyl sulfoxide (passaged less than 10 times) were thawed, washed in DMEM, centrifuged, and resuspended in DMEM. Ten thousand cells were plated on a coverslip and incubated at 37°C for 1 to 3 days. The cells were covered with colchicine and incubated for 2 hours. The cells were fixed with acetic acid/methanol (1:3) and banded and stained with trypsin–Leishman solution. Fifteen to 30 cells were analyzed from each cell line.

Radial Dish Motility Assay

A simple technique was previously developed in our laboratory to evaluate specifically in vitro cell locomotion from a region of high cell density (as in bulky tumor) to a region of lower cell density (as in surrounding brain). Twenty thousand human brain tumor cells, suspended in 1 ml of feeding medium (DMEM with 15% FCS), were plated in the center of a circular petri dish 8 cm in diameter. After 12 hours at 37°C, in a humidified 5% CO₂ atmosphere, the medium was discarded, establishing a 2-cm circular zone of plated cells in the center of the petri dish. The attached cells were washed and then fed with 10 ml of feeding medium. Motility was determined by counting daily (in triplicate) the number of cells per random high-power field (× 20) at predetermined distances (0–1, 1–2, and 2–3 cm) from the perimeter of the central zone. The area of each concentric ring field of cells increases as the distance from the center of the petri dish increases. To calculate the total number of cells in each concentric ring from the mean number of cells counted per high-power field, the surface area of each ring (which increases with increased distance from the central zone) was used to determine cell density. The average distance traveled each day by the cells was computed by multiplying the number of cells (the mean of the three cell counts) in each concentric ring. Each count was “weighted” relative to the area of its respective ring to determine cell density.

Statistical Analysis

Statistical analysis was performed using a simple linear regression analysis to generate the best-fit line of the average distance traveled plotted against time. The slopes with standard errors for each best-fit line were then compared using a t-statistic to determine statistical significance.

Sources of Supplies and Equipment

The C6 rat astrocytoma cell line was obtained from American Type Culture Collection, Rockville, MD. The GFAP immunoperoxidase staining kit, Endo/Blocker, and the 1× Automation Buffer were obtained from Biomeda Corp., Foster City, CA. Scott’s Tap Water was provided by Sigma Chemical Co., St. Louis, MO, and the sea plaque agarose was obtained from FMC Bioproducts, Rockland, ME. Fisher Scientific, Pittsburgh, PA, provided the Hauser Brightline hemocytometer.

Results

Histopathology and Morphology of Surgical Specimens and Cell Cultures

Cell lines were derived from gross tumor and from histologically normal brain that was more than 4 cm distant from the bulk tumor (Figs. 1 and 2). Although the evaluation of frozen sections of histologically normal brain specimens revealed no tumor cells, histological evaluation after fixation and staining (using hematoxylin and eosin and GFAP) demonstrated scattered cells that were deemed suspicious for tumor in one of the four cases (a glioblastoma multiforme). The other three cases showed no evidence of tumor cells. Three of the tumors were Grade IV astrocytomas (glioblastomas multiforme); one was a Grade III oligodendroglioma. All three glioblastoma multiforme gross tumor specimens were GFAP positive; the oligodendroglioma was GFAP negative.

Evaluation using phase-contrast microscopy and light microscopy (with hematoxylin and eosin and with GFAP) demonstrated that only one of the gross tumor–histologically normal brain cell culture pairs showed morphologi-
cal differences between the cells derived from gross tumor and those from histologically normal brain (Figs. 3 and 4). All eight cultures showed anaplastic cells, which is typical for glioma cells (not normal glia) in culture.

**Growth and Growth Rates of the Gross Tumor and Histologically Normal Brain Cultures**

Each of the eight cultures was initiated without difficulty. Initial passage and expansion (cells were diluted 1:3) were successfully completed within 7 days for each of the cultures. Each of the cell lines was passaged at least 10 times (minimum 10, maximum 46). In all three cases of glioblastoma multiforme, the growth rates of cultured cells from histologically normal brain were greater than the growth rates of the cultures cells from gross tumor. The oligodendroglioma culture pair had opposite results, with the gross tumor cells growing faster than those derived from the histologically normal brain (Table 1).

**Anchorage-Independent Growth in Soft Agarose**

All eight cell cultures grew well in soft agarose, with cell colonies clearly seen 14 days after plating in tubes.

**Chromosomal Analysis**

Karyotype analysis of seven of the eight cultures (the eighth became contaminated and was no longer available for study) confirmed that these cell lines had abnormal karyotypes consistent with a neoplastic origin.

**Radial Dish Assay Determination of Tumor Cell Motility**

Motility, determined as the slope of the line formed from the average distance traveled plotted against time (mean distance in millimeters traveled per day), demonstrated statistically equal rates for gross tumor and histologically normal brain tumor cells (Table 1).

**Discussion**

**Clinical Course of Patients With Glioblastoma Multiforme**

Malignant gliomas are the most common primary supratentorial cerebral neoplasms in adults. These tumors are diffusely infiltrative and rapidly fatal. Despite advances in brain tumor therapy, little impact has been made on the survival in patients with malignant gliomas, which confer a median survival time of 12 to 14 months. It is the motile invading cells from these tumors, which cannot be surgically extirpated, that are responsible for glioma recurrence following radical resection. In addition, by unknown mechanisms, these cells can lead to progressive neurological dysfunction without evidence of mass effects or recurrence of bulk disease. Although current treatments are focused on tumor control within the gross tumor and/or on the area of brain adjacent to the gross tumor, it is now clear that malignant glioma cells invade much of the neural axis prior to diagnosis. Although it was assumed that patients with malignant gliomas die from either tumor mass or complications of treatment, it has been shown that a significant portion succumb to generalized neurological deterioration referable to areas of the brain far from the gross tumor. Furthermore, many patients with brain tumors die without any significant bulk tumor present.

**Culturing Tumor Cells From Histologically Normal Brain**

To elucidate the biological attributes of glioma cells that have invaded histologically normal brain, we established these cells in culture for in vitro investigation. Clearly not all of the cells from these brain specimens were neoplastic. However, neoplastic cells have significant growth advantages over nonneoplastic cells that allow them to grow and proliferate in vitro. Thus, the ability of these cells to grow and divide in vitro is essential for further investigation.
Isolated invading glioma cells

Fig. 4. Photomicrographs showing cultured histologically normal brain (A) and gross tumor (B) glioblastoma cells. As opposed to the other three gross tumor–histologically normal brain culture pairs, this pair shows distinct morphological differences between the tumor cells derived from gross tumor and those from histologically normal brain. Gross tumor cells are spindlelike in appearance, whereas the histologically normal brain cells are more typical of cultured human glioblastoma cells. H & E, original magnification × 10.

The doubling time for normal glia in culture is several days, which is much slower than any of the eight cultures. Furthermore, initiation of normal astrocytes in culture is difficult and often unsuccessful, whereas these cultures were relatively easy to initiate and maintain for many passages. The in vitro motility rates for the eight cultures investigated were consistent with the rate for malignant glial cells. We demonstrated previously that normal cultured human astrocytes have very low in vitro motility, much lower than any of the cell cultures studied in this investigation. Chromosomal analysis of seven of the eight cultures confirmed that these cell lines had neoplastic karyotypes. Based on these findings, we conclude that human gliomas grow invasively, with tumor cells demonstrable over 4 cm from the gross tumor. Furthermore, a standard histological evaluation of histologically normal brain for the presence of individual neoplastic cells is insensitive compared to a histological evaluation using cell culture. Therefore, intraoperative frozen-section examination of glioma resection margins using hematoxylin and eosin and GFAP staining may be misleading.

Growth Rate Determinations of Cells Cultured From Gross Tumor and Histologically Normal Brain

Previous studies in our laboratory demonstrated that prelabeled C6 glioma cells that invade the contralateral hemisphere of rats maintain their tumorigenicity. These cells can be grown in culture and form gross tumors when implanted into naive rats. In the present study, we found that cells derived from histologically normal brain grew faster in three of the four gross tumor–histologically normal brain culture pairs, corroborating the stem cell capacity of these invasive cells. All eight cultures grew faster than normal glial cultures. It is possible that although invading cells do enter mitosis, the daughter cells move away from each other. Alternatively, the cells may not enter mitosis while motile. Observation of glioma cells in vitro, using time-lapse videomicroscopy, has shown that locomotion and mitosis are not mutually exclusive processes. However, invasion requires glioma cell interaction with the surrounding brain, which may impede the initiation of mitotic events.

Glioma Cell Motility

Glia exhibit extensive cellular movement during development of the central nervous system and in response to injury to that system. The normal motility exhibited by glia is retained and amplified in neoplastic glia. The locomotion exhibited by glia and glioma cells is ameboid, with ruffling leading cell edges and formation of pseudopodia demonstrable with time-lapse video microscopy. To assess the in vitro motility of glioma cells, our laboratory previously developed and modified a number of experimental models. Time-lapse videomicroscopy recordings of plated brain glioma cells revealed a motility rate of 12.5 μm/hour with an ameboid form of locomotion. The radial dish assay was devised to evaluate motil-
ity quantitatively, comparing a region of high tumor cell density (as in the gross tumor) to one of lower tumor cell density (as in the histologically normal brain). Using this in vitro model, cells were found 1 cm from the perimeter by 24 hours and 3 cm from the perimeter by 4 days. Different human brain tumor cell lines exhibited varying motility. Increasing the serum concentration increased motility. Coating the polystyrene petri dish with fibronectin did not alter motility. Arrest of the cells in the G₀ phase by administration of hydroxyurea did not alter cell motility. Addition of cytochalasin B, which is used to block cytoskeletal assembly, prevented cell motility. Taxol, a chemotherapeutic agent that is currently being developed for use with gliomas and that induces formation of a metastable cytoskeleton, increased glioma motility. Subpopulations of cells were created by clonal amplification of cells that had migrated to the dish periphery. Although morphologically indistinguishable when compared to the original cell line from which they were derived, these subpopulations demonstrated significantly increased motility. In vivo studies in rodents revealed extensive migration of pre-labeled C6 rat astrocytoma cell xenografts implanted in the rat forebrain. Within 7 days postimplantation, astrocytoma cells were found throughout the brain. Of note, the only area in which bulk disease was identified was at the site of injection; distant cells remained single.

On the basis of the previous findings we have described, we hypothesized that tumor cells cultured from histologically normal brain would represent a subpopulation of glioma cells with increased motility. However, measured motilities of the cells from gross tumor and histologically normal brain were equivalent. It is possible that the invasive phenotype is induced by the local milieu rather than specific genotypic alterations. It has been demonstrated in vitro, using a modification of the radial dish assay, that specific cytokines alter the rate and direction of glioma cell movement. Thus, local cerebral cytokine concentrations may lead to changes in glioma invasion. It is even possible that increased production of cytokines by the injured brain at the site of the surgical resection may be both mitogenic and chemotactic to the tumor cells that have invaded the histologically normal brain, in part explaining why tumor recurrence tends to take place at (or near) the resection margin. The finding of equivalent motilities of gross tumor and histologically normal brain cells within each cell culture pair, with different motilities among the gross tumor–histologically normal brain pairs, again argues that the histologically normal brain cultured cells are tumor cells rather than normal glia (which have a very low rate of in vitro motility, as determined by the radial dish assay).

Most neurooncology research has focused on slowing the growth of gross tumor. Although recent laboratory investigations have begun to address mechanisms underlying tumor invasion, this current study demonstrates that malignant gliomas have invaded distant, histologically normal brain by the time of diagnosis. Therapies that only address gross tumor lessen the likelihood of lethal mass effects, but do not offer assistance to the large number of patients dying from other causes. Therapies that do not eradicate bulk disease predispose toward herniation as a cause of death. Clearly, therapeutic strategies must be developed that are directed at eradication of invasive glioma cells as well as the gross tumor. To evolve pertinent effective therapeutic modalities, tumor cells in histologically normal brain must be biologically characterized and then specifically targeted. Evaluation of new treatment modalities for malignant gliomas should assess the impact of the therapy on tumor cells in distant, histologically normal brain as well as in the gross tumor.

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