Growth of precultured human glioma specimens in nude rat brain

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Object. The aim of this study was to develop an improved animal model for brain tumor study. The need for better and more relevant brain tumor models is generally acknowledged. Glioma tissue can be cultured directly from the biopsy specimen as tumor spheroids. Using such precultured tissue, a new in vivo model for studying human gliomas was established.

Methods. Precultured small tumor spheroids (< 300 μm) prepared from cell lines or tumor biopsy fragments were injected into the brains of immunodeficient rats by using a 5-μl Hamilton syringe that had a piston in the needle. Tumors could be established by injecting a single spheroid derived from the U-87MG cell line, whereas inoculation of 10 spheroids resulted in a tumor take comparable to that attained with injection of 10^6 single cells. Biopsy specimens obtained from six patients who underwent surgery for glioblastoma multiforme were cultured as organotypic spheroids for 11 to 18 days before inoculation into the rats. The animals were killed 3 months after spheroid implantation. Microscopic examination revealed tumor growth in 87.5 to 100% of the animals inoculated with tumor spheroids from all but one of the tumor biopsy specimens. Extensive invasion and cell migration along the nerve tracts of the corpus callosum was found in tumors that originated from four of the six biopsy specimens.

Conclusions. This approach, in which spheroids from precultured biopsy specimens are injected into the brains of immunodeficient animals, provides new means for experimental studies of human malignant brain tumors in a clinically relevant animal model.

KEY WORDS • neoplasm invasiveness • glioma • animal model • orthotopic xenograft • spheroid culture • rat

INVASIVENESS and diffuse infiltrative growth are hallmarks of brain tumors of glial lineage. Human glioma cell lines are relatively easily established from their original tumors and can be grown in both in vitro culture and as subcutaneous tumors in immunodeficient animals.11,13 Glioma cell lines inoculated into the brains of these animals often result in tumor growth, but because of the clonal origin of the cell lines the resulting tumors may not be fully representative of the original lesions in patients.10 Tissue obtained from human glioma at biopsy has also been inoculated directly into the brains of immunocompetent rats, and a fraction of the specimens have given rise to large, diffusely growing tumors after 3 months.15

Although the brain is regarded as a partly immuno-privileged site, the tumor take may be reduced because of local immune response in immunocompetent animals. This problem may be circumvented by using immunodeficient animals, as indicated by the high tumor take reported in previous studies in which nude mice were used as hosts.7,14 but freshly dissected tumor tissue can be difficult to obtain. Moreover, immediate transplantation into animals is not always feasible and does not allow for testing of the viability of the selected tumor specimens. Alternatively, fresh brain tumor tissue cultured as organotypic spheroids in vitro may be used; these have been shown to retain the morphological properties and characteristics of the original tumor.3 Furthermore, when such organotypic tumor spheroids were added to fetal brain cell aggregates, the in vitro growth pattern seemed to reflect the in situ invasive behavior of the original brain tumor.4

With the aim of developing an in vivo model representative of human brain tumors, we have used a new technique for injection of tumor spheroids into the brains of immunodeficient nude rats and used this method to establish tumor xenografts from precultured fragments of human brain tumor biopsy specimens.

Materials and Methods

Culture of Tumor Cell Lines

The U-87MG cell line was cultured in Dulbecco’s modified
Eagles medium (DMEM), supplemented with 10% fetal calf serum, glutamax, and four times the prescribed concentration of nonessential amino acids. The cells were detached using 0.01 M ethylenediamine tetraacetic acid, and spheroids were initiated by seeding approximately 2 × 10^6 cells into an 80-cm² culture flask base coated with 0.75% agar in DMEM, and cultured using 15 ml of overlay culture medium. The cells clustered and formed spheroids, and 1- to 2-week-old spheroids were used in the experiments.

**Culture of Tumor Tissue**

Tissue obtained from the brain tumors of patients undergoing craniotomy for glioblastoma multiforme (GBM) was cut into small fragments and cultured according to the method described by Bjerkvig, et al.³ Briefly, the tumor tissue was dissected at surgery and transferred to tissue culture medium. Macroscopically representative tissue from the tumor was then selected for culture and for histopathological diagnosis. The specimens were minced with a scalpel and placed in 80-cm² tissue culture flasks base coated with 0.75% agar in DMEM. The fragments were cultured until they formed spheroids. The volume of the overlay suspension was 1.5 ml DMEM supplemented with 10% heat-inactivated fetal calf serum, four times the prescribed concentration of nonessential amino acids, 2% t-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). The flasks were kept in a standard tissue culture incubator (100% relative humidity, 95% air, and 5% CO₂).

**Animal Preparation**

All procedures and experiments involving animals in this study were approved by The National Animal Research Authority and conducted according to the European Convention for the Protection of Vertebrates Used for Scientific Purposes. Nude rats (Han: nu/nu Rowett) were bred in our rodent facility. Animals were kept at 25°C in a specific pathogen-free environment, in positive-pressure rooms with filtered and humidified air (55% relative humidity) on a standard 12-hour night and day cycle. Four- to 5-week-old rats weighing 60 to 80 g were used in these experiments. At the time of the experiments, the rats were anesthetized by subcutaneous injection of 0.1 mg/kg fentanyl, 5 mg/kg fluanisone, and 2.5 mg/kg midazolam.

**Intracranial Inoculation of Tumor Spheroids**

A 5-μl Hamilton syringe in which the piston reached the tip of the needle was used, with the needle ground to an angle of 30°. The syringe was washed with 70% alcohol and flushed with sterile saline before and with sterile water after use. Cultured brain tumor spheroids ranging in diameter from 200 to 300 μm were selected using a micropipette and a stereomicroscope with a calibrated reticle in the eyepiece. Ten spheroids were aspirated into the syringe with the aid of an operating microscope, placed in the syringe holder of a small-animal stereotactic frame, and a burr hole was drilled 1 mm to the right of the midline fissure and 1 mm posterior to the frontal fissure of the animal’s skull by using a dental drill. The tip of the syringe was then placed 2.5 mm below the surface of the brain, and 5 μl DMEM without serum containing the 10 tumor spheroids was slowly injected into the brain, the needle was slowly withdrawn, and the skin wound was closed with wound clips.

Three months later, the animals were anesthetized by subcutaneous injection of a mixture of fentanyl, fluanisone, and midazolam and killed by intracardial injection of pentobarbital. The brains of the animals were removed and fixed in 4% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin in preparation for light microscopic examination.

**Determination of Ki-67 Labeling Index**

The tissue sections were deparaffinized, rehydrated in decreasing concentrations of ethanol and washed in phosphate-buffered saline. The sections were then boiled in a microwave oven four times in 0.01 M citrate buffer, pH 6, for 5 minutes each time. After the blocking step, the sections were covered with a 1:100 dilution of anti-Ki-67 monoclonal antibody in phosphate-buffered saline and incubated in a humidity chamber for 30 minutes at room temperature. The primary antibody was visualized using a standard indirect avidin-biotin-peroxidase technique. The sections were counterstained with hematoxylin and eosin.

Using × 40 magnification, 10 examined fields of the stained slides were stored as color computer images and analyzed using an image-analysis program. The labeled nuclei were counted manually on the computer screen, followed by computerized discrimination and counting of all the cells in the stored image. The fraction of peroxidase-labeled cells divided by the total number of cells in the image was calculated. The average Ki-67 labeling index was calculated based on the cell counts in 10 images of human GBM biopsy specimens and in four images of the animal brain tumors.

**Sources of Supplies and Equipment**

The DMEM was purchased from BioWhittaker, Verviers, Belgium, the fetal calf serum from Biological Industries, Kibbutz Beit Haemek, Israel, and the glutamx from Gibco BRL, Paisley, Scotland. The culture flasks and agar (Agar Noble) were obtained from Nunc, Roskilde, Denmark, and Difco Laboratories, Detroit, MI, respectively. The syringe was supplied by Hamilton, Bonaduz, Switzerland, and the small-animal stereotactic frame was purchased from Kopf, Tujunga, CA. The anti-Ki-67 monoclonal antibody (A047) was purchased from DAKO, Glostrup, Denmark, and the Vectastain for avidin-biotin-peroxidase staining from Vector Laboratories, Burlingame, CA. The image-analysis program (NIH Image, Version 1.62b4/fat) was obtained from the National Institutes of Health, Bethesda, MD.

**Results**

**Growth of U-87MG Cells in Vivo**

After injection of a single-cell suspension of 10⁶ cells into the nude rat brain, all eight animals exhibited symptoms of fatigue, weight loss, and/or hemiparesis and were killed after a mean of 22.8 days (Fig. 1A). A reduction in the number of injected cells to 5 × 10⁴ resulted in an increase in the symptom-free interval of the animals to 30 days, similar to that seen in the animals injected with 10 tumor spheroids.

Injection of spheroids instead of single-cell suspensions resulted in symptom-free intervals of 32.3 days with 10 spheroids, 38.5 days with five spheroids, and 46 days when only one spheroid was injected (Fig. 1B). All animals, four in each group, showed symptoms of tumor growth.

At autopsy, all U-87MG tumors were found to be large and compressing the rat brain tissue. The tumors remained localized at the site of injection, and little invasion into the normal tissue was observed (Fig. 2). No histopathological differences were observed between tumors established from single-cell suspensions and spheroids.

**Growth of Tumor Spheroids Established From Biopsy Specimens**

Biopsy specimens obtained at surgery in six patients with intracranial tumors were minced into fragments and cultured in vitro as described. All tumors formed multiple spheroids from the cultured fragments. The time in culture before inoculation varied from 11 to 18 days for the different tumor types (Table 1). Based on experiments with the U-87MG cell line, 10 tumor spheroids were injected into the brain of each rat. None of the rats developed symptoms of intracranial neoplastic growth during the 3-month observation period.
At autopsy, however, tumor take was found macroscopically in animals injected with spheroids from five of the six GBMs. The fraction of rats with macroscopically detectable tumor xenografts varied from 37.5 to 100% between the five tumor types (Table 1). Histopathological examination revealed tumors in all rats inoculated with spheroids prepared from these five GBMs, except in one of the rats that received GBM Case III spheroids. Tumor spheroids from GBM Case V did not form tumors in any of the inoculated rats. A lesion that was diagnosed as a tumor on macroscopic observation in one of the rat brain injected with GBM Case V was actually a large cyst, as seen on histopathological examination. In three other rats in this group, the incision channel contained a few tumor cells.

The detected tumors differed with respect to cellularity, growth pattern, and invasiveness (Table 1). Some of the lesions were widely disseminated, invading and destroying the normal architecture of the corpus callosum and adjacent structures (Fig. 3), whereas other tumors were less invasive. In rats inoculated with spheroids from GBM Case III, diffusely seeded Ki-67-positive cells were found in both hemispheres, showing an extensive spread of the tumor cells. For all the biopsy specimens except GBM Case IV, tumor cell migration was found along the fiber tracts of corpus callosum and into the contralateral hemisphere, but no invasion into the meninges or the calvarial bones was observed. Even when the tumors were large and caused asymmetry of the hemispheres, none of them crossed the midline by bridging the sagittal fissure directly, rather migration was seen along the corpus callosum to the contralateral hemisphere.

**Morphological Appearance of Biopsy Specimens and Corresponding Rat Brain Tumors**

All biopsy specimens consisted of cell-rich tumors with a pleomorphic appearance showing slightly different cellular characteristics among the different lesions. Necrotic areas typical for GBM were found in all the biopsy samples, as well as endothelial proliferation, swelling, and thrombosis. In addition, GBM Case IV tumors showed groups of small dark cells with condensed chromatin and scarce cytoplasm. Cells circling the capillaries (perivascular pseudorosettes) resembling an ependymoma cell component were also found in this tumor. In GBM Case V, a large number of the cells showed astrocytic differentiation (gemistocytes) in large areas of the tumor in addition to the previously described characteristics.

Importantly, the same basic cellular appearance and organization found in the biopsy samples were present in
the corresponding rat brain xenografts (Fig. 4). In GBM Case III, the appearance of the xenograft closely resembled the ependymoma-like cell component of the biopsy specimen. In addition, all the tumors originating from this biopsy sample were sharply demarcated from the surrounding brain tissue. However, necrosis and endothelial cell proliferation were never seen in the rat brain tumors.

**Comparison of Ki-67 Labeling Index in Biopsy Specimens and Rat Brain Tumors**

The Ki-67 labeling index was determined both in the original biopsy specimens and in the corresponding nude rat brain tumors (Table 2). Whereas the labeling index in the biopsy tissue ranged between 0% and 9%, it was higher in the xenografts, with values from 13 to 34%. The labeling index also varied within the different fields in the individual tumors investigated, as indicated by the minimum and maximum values given in Table 2. However, the index in the original lesion was always lower than in the rat brain tumor. In GBM Case VI, no Ki-67–positive tumor cells could be seen in one part of the specimen, whereas another part contained the indicated number of positive cells. This part of the tumor differed in histopathological appearance from the other, with part of the lesion showing fewer pleomorphic cells and generally fewer cells in the specimen.

**Discussion**

Various animal models have been used for studies of brain tumor biology and response to experimental therapy, as reviewed in Schold and Friedman and Peterson, et al. Nevertheless, the need for better and more relevant models for the study of human malignant brain tumors is generally acknowledged. We report the growth of tumors in the brains of immunodeficient animals, thereby reducing to a minimum the incidence of malignant cell seeding along the injection tract. In animal model experiments with the U-87MG cell line we first showed that tumor cell spheroids can be used reliably to establish tumors in the brains of nude rats. Even inoculation of a single U-87MG spheroid was sufficient to obtain tumor growth in each of the four animals in this group but at the cost of a somewhat more variable latency time before clinical symptoms emerged. Previously, spheroids cultured from a rat glioma have been used as a source of tumor tissue in an animal brain tumor model. However, this and other studies not based on injection of single-cell suspensions required the use of invasive operative techniques. Such techniques are relatively laborious and time consuming, and this may prevent widespread use of such methods. In contrast, the procedure for injection of three-dimensional spheroids described in this study is simple and reliable. This method may be of value not only for establishing orthotopic growth of human brain tumors but also for implantation of spheroids prepared from other tumor types to mimic metastatic growth at a biologically relevant site.

Variability in tumor take may be troublesome, particularly if the model system is intended for treatment studies, in which a consistent tumor take is a prerequisite. In our work with spheroids from clinical specimens, macroscopic examination in some cases failed to reveal tumor formation. With the exception of spheroids cultured from GBM Case V, established tumors were detected by using microscopy in nearly 100% of the cases, an indication that a large proportion of the cells in the spheroids are capable of forming spheroids. Selection of specific cell populations may therefore be less of a problem, which is important when such brain tumor xenografts are used for biological studies. The high tumor take makes this model system suitable even for evaluating effects of experimental therapy.

The cultured cells from GBM Case V formed spheroids in vitro, but these did not grow into tumors in the animals. Although half of the biopsy specimen contained areas with Ki-67–positive cells, astrocytic differentiation was another predominant feature of the specimen, possibly indicating a limited tumorigenic potential. We might speculate whether tumor spheroid formation can take place without the presence of proliferating (Ki-67–positive) cells, but such spheroids may have a low capacity for tumor establishment in vivo. Prior to inoculation in immunodeficient animals, microscopic examination and Ki-67

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**TABLE 1**

**Macroscopic and microscopic appearance of rat brains inoculated with precultured human biopsy specimens**

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Days in Culture</th>
<th>Take</th>
<th>Macroscopic Appearance</th>
<th>Take</th>
<th>Microscopic Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBM I</td>
<td>11</td>
<td>5 of 8</td>
<td>2 large white diffuse tumors, 3 small tumors</td>
<td>8 of 8</td>
<td>increased cellularity, diffuse spread of cells in brain tissue w/ 1 example of tumor cells migrating along corpus callosum; abundant capillaries cellular tumors, small cells, diffusely migrating, especially along corpus callosum</td>
</tr>
<tr>
<td>GBM II</td>
<td>14</td>
<td>3 of 8</td>
<td>3 brains w/ diffusely enlarged hemispheres</td>
<td>8 of 8</td>
<td>cellular tumors w/ rich capillary network, small cells, &amp; invasion into surrounding tissue &amp; corpus callosum</td>
</tr>
<tr>
<td>GBM III</td>
<td>11</td>
<td>5 of 8</td>
<td>diffuse, large white tumor in 2 animals, 3 w/ smaller tumors</td>
<td>7 of 8</td>
<td>small dark cells surrounding large necrotic areas, clearly demarcated from surrounding tissue &amp; not invasive in corpus callosum</td>
</tr>
<tr>
<td>GBM IV</td>
<td>12</td>
<td>7 of 7</td>
<td>4 brains w/ large tumors, 3 w/ small but visible tumors</td>
<td>7 of 7</td>
<td>1 large cyst at injection site in macroscopically visible tumor; injection site w/ few remaining tumor cells detected in 3 rat brains</td>
</tr>
<tr>
<td>GBM V</td>
<td>18</td>
<td>1 of 8</td>
<td>1 large “tumor” detected</td>
<td>0 of 8</td>
<td>diffusely growing tumors in hemispheres, w/ massive invasion &amp; migration along corpus callosum; abundant vascularization</td>
</tr>
<tr>
<td>GBM VI</td>
<td>12</td>
<td>8 of 8</td>
<td>large white tumors in all brains, diffuse growth; 3 tumors giving rise to brain displacement</td>
<td>8 of 8</td>
<td></td>
</tr>
</tbody>
</table>
staining of the biopsy specimen and/or precultured spheroids may reveal whether in vivo experiments will be successful, thereby serving as a tool for selecting specimens with the capacity for in vivo growth.

Most previous in vivo studies of brain tumor growth and invasiveness have involved the use of permanent cell lines inoculated into various sites in the animals. Although such systems may be highly standardized and have proven
Glioma biopsy specimens in nude rat brain

**TABLE 2**

*Ki-67 labeling index in biopsy specimens and in corresponding tumors growing in nude rat brain*

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Biopsy Specimen</th>
<th>Tumor in Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ki-67 Labeling Index</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average % Labeled Cells (min–max)</td>
<td>Total Counted</td>
</tr>
<tr>
<td>GBM I</td>
<td>7 (2–17)</td>
<td>1354</td>
</tr>
<tr>
<td>GBM II</td>
<td>6 (4–9)</td>
<td>2552</td>
</tr>
<tr>
<td>GBM III</td>
<td>4 (2–7)</td>
<td>1754</td>
</tr>
<tr>
<td>GBM IV</td>
<td>6 (3–8)</td>
<td>3971</td>
</tr>
<tr>
<td>GBM V</td>
<td>3 (0.6–7)†</td>
<td>1687</td>
</tr>
<tr>
<td>GBM VI</td>
<td>9 (6–11)</td>
<td>3246</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent the minimum and maximum percentage of cells labeled. Abbreviation: ND = not done.
† Labeling index in the part of tumor containing Ki-67–positive cells.

useful for specific purposes, cell line–based tumors may not reflect the cellular heterogeneity of the parent tumor. The most widely used nonhuman brain tumor lines, represented by the C6 glioma, 9 L gliosarcoma, BT4 A, RG2, and the T9 glioma cells, were all established using in vivo carcinogenesis.1,2,8,16 All of these cell lines are of animal origin and most likely represent selected variants with a low degree of intercellular heterogeneity. In contrast, human GBMs are well known for their heterogeneity, and cultured spheroids prepared from biopsy specimens have been shown to retain the cellular complexity, DNA ploidy, and stromal elements present in the original tumor in vivo.7 Therefore, the use of such spheroids may better mimic human gliomas than cell lines from other species.

It has been shown that spheroids from C6 glioma inoculated into Wistar rats form localized tumors, with a sharp margin toward normal brain tissue,5 whereas other researchers have found such cells to be invasive.14 As discussed in the latter study, these differences may be more dependent on the host tissue, for example, the rat strain used in the experiments, than the type of tumor cells used for the inoculation. In our study, the human U-87MG cells did not invade but compressed the brain tissue. No difference in the histopathological appearance of the U-87MG tumors was observed whether the tumor cells were inoculated in the form of spheroids or in a cell suspension. For another human brain tumor cell line, D-54MG, the borderline between the malignant and normal tissue was more diffuse than that seen with U-87MG (data not shown).

As previously emphasized in other studies, tumors xenografted directly into the animal model from the human tumor show histological features more similar to the original lesion than those originating from established glioma cell lines.13 Furthermore, the lesions that develop from implanted organotypic spheroids obtained from brain tumor biopsy procedures seem to have maintained important features of the original tumor and should be more representative of clinical glioblastomas than the tumors originating from cell lines. Thus, all tumors originating from biopsy specimens, except those seen for GBM Case IV, showed extensive invasion into the brain tissue, in contrast to the findings with glioma cell lines. Tumors originating from GBM Case IV showed no invasion into the brain tissue. On histopathological examination we found that this tumor contained an ependymal cell component, and this might have dominated the implanted spheroids, as reflected in the morphological appearance of the nude rat tumors. Other researchers have investigated migration patterns of injected cells and have shown that migration along the corpus callosum may occur after implantation of a suspension of human tumor cells and also of fetal brain cells.9 However, the widespread and extensive dissemination of tumor cells derived from biopsy samples observed in our study is exceptional, and we emphasize the clinical challenges presented by GBM.6

**Conclusions**

In summary, we have developed a model system in which brain tumors are established with a high take rate after a simple injection of a precultured organotypic biopsy specimen. This approach should be useful for studies of the biology of human gliomas, as well as for assessing the effects of established and new therapeutic alternatives in this treatment-resistant malignancy.

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**References**


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