A brain-tumor model utilizing stereotactic implantation of a permanent cannula

VITTORIO M. MORREALE, M.D., BARBARA H. HERMAN, PH.D.,
VIOLETTA DER-MINASSIAN, B.S., MIKLÓS PALKOVITS, M.D., PHILLIP KUBES, PH.D.,
DAVID PERRY, PH.D., ATILÁ CSIFFARY, D.D.S., AND ALEX P. LEE, B.A.

Brain Research Center, Children's National Medical Center, Washington, D.C.; Departments of Psychiatry and Behavioral Sciences, Pediatrics, and Pharmacology, The George Washington University Medical Center, Washington, D.C.; and Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, Maryland

A tumor model involving stereotactically implanted culture-reared tumor cells is presented. Stainless steel cannulas were stereotactically and permanently implanted into the caudate nucleus of 30 rats. The animals were separated into two groups. In Group I, 15 animals received a 10-μl injection containing 10^6 C6 glioblastoma cells (five rats), 10^4 Walker 256 breast carcinoma cells (five rats), or cell medium (five rats). The coordinates were A(+1.5), L(+3.0), and DV(-5.0). In Group II, the coordinates were changed to A(+1.0), L(+3.0), and DV(-5.0) and the same number of rats received a 1-μl injection containing 10^5 cells of each tumor in an attempt to produce more focal tumors. Two weeks after implantation, brain sections were stained with cresyl violet and a subset was stained for glial fibrillary acidic protein (GFAP). A computerized morphometric analysis system was used to quantify tumor size. In Group I, the mean C6 tumor areas ± standard error of the mean at specific coordinates were (in sq mm): A(+4.7) 0.4 ± 0.2, A(+3.7) 3.5 ± 1.1, A(+2.7) 5.7 ± 1.7, A(+1.7) 9.5 ± 2.3, A(+0.7) 7.5 ± 3.2, A(-0.3) 3.7 ± 2.9, and A(-1.3) 0.3 ± 0.3. A nearly identical tumor mass and extension into the brain was produced in rats injected with Walker 256 cells. Similar C6 tumour areas were indicated in adjacent sections stained with cresyl violet and GFAP. Tumor was found in the caudate nucleus in all 10 rats, but not in the nucleus accumbens, fornix, or hippocampus. In Group II animals, tumor magnitude and extension into the brain were greatly reduced. The 10^6 cells in the 10-μl volume was the most reliable tumor load for obtaining uniform tumors in different animals. The similarity of tumor distribution across different animals was indicated by the low variance of tumor area at specific anteroposterior coordinates. Reproducible and well-circumscribed caudate nucleus tumors were produced using this stereotactic procedure.

KEY WORDS • astrocytoma • brain neoplasm • C6 glioma • tumor cells • glioblastoma multiforme • stereotaxis

The median survival time of individuals diagnosed as having malignant brain tumors is usually less than 1 year. Therefore, it is necessary to develop reliable and precise models of brain tumors in animals in order to understand factors that modulate the growth of brain tumors and to develop treatments that inhibit these tumors. A variety of brain-tumor models have been reported. Models vary with respect to animal host, type of tumor used, method of tumor production, location of tumor, size of tumor, and tumor growth time. A number of chemically induced tumors have been studied, including C6 glioma (which is a malignant astrocytoma derived from chemically induced rat astrocytes), schwannoma, and others. Numerous types of chemicals have been used to induce brain tumors in rats.

A variety of routes of administration of carcinogenic substances to animals have been utilized to induce brain tumors, including oral, subcutaneous, intravenous, and intracerebral. One method for producing brain tumors in rats is by injecting tumor cells into the cerebrovasculature. Ushio, et al., injected W256 carcinoma cells into the carotid artery of rats; however, intracerebral growth yields were poor (about 25%), and massive extracerebral metastases were common. The next development was the intracisternal injection of W256 carcinoma cell suspensions into rats, which produces a widespread meningeal carcinomatosis. A more precise approach involves the free-hand injection of tumor cells into particular areas of rat brain, but this method has been criticized for its lack of reliability, poor intracerebral growth yields, and rapid spread to...

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extracranial tissue. Some free-hand injection methods in which neoplastic glial cells are injected into the frontal cortex of rats do yield a 100% tumor take; however, even this method results in extracerebral masses in about one-half of the animals. Finally, Kobayashi, et al., found that stereotactic implantation of tumor into the rat brain (caudate nucleus) resulted in a more reliable tumor model, with a 99% to 100% yield of intracerebral tumor as well as a marked reduction in extracranial extension.

We present a stereotactic procedure for the implantation of a discrete number of cultured tumor cells (malignant rat astrocytes or rat breast carcinoma) into the caudate nucleus of the rat brain. Our model offers a further refinement of the method described by Kobayashi, et al., by using a permanently implanted stainless steel cannula to decrease the spread of tumor along the injection line. A computer morphometric analysis of tumors offers a precise and objective method for quantifying the spread of tumor into the brain.

Materials and Methods

Tumor Cells and Hosts

Thirty male Wistar rats, each weighing 200 to 250 g, were used for this study. Rats were individually housed in Plexiglas cages and given free access to standard laboratory rat chow and water. The room was maintained on a 12-hour day/night cycle at 22.2°C.

The donor C6 cells were 40th-passage C6 glioma-astrocytoma cells obtained as a culture. This cell line is derived from N-methyl-N-nitrosourea-transformed rat astrocytes. The donor Walker 256 cells were 65th-passage LLC-Walker 256 rat breast carcinoma cells obtained as a culture. Cell culture was grown by W. J. Goldberg, Ph.D., according to the method of Bernstein, et al.

Mounting the Guide Cannula

The rats were deprived of food and water 12 hours before surgery. They were deeply anesthetized with pentobarbital (Nembutal), 42 mg/kg, by intraperitoneal injection. The head was mounted into a stereotactic head holder in a flat-skull position determined by equalizing the dorsal-ventral (DV) distance (within 0.05 mm) at the intersection of the bregma at the midline and the lambda at the midline. A burr hole was made using a 1.4-mm diamond-tipped burr at the following coordinates: Group I: anterior 1.5 mm from bregma (A(+1.0)) and lateral 3.0 mm relative to the mid sagittal sinus (L(+3.0)); Group II: A(+1.0) and L(+3.0). For each rat, a stainless steel No. 22 guide cannula was implanted into the right caudate nucleus so that the tip was at the following coordinates: Group I: A(+1.5), L(+3.0), DV 5.0 mm from skull top (DV(-5.0)); Group II: A(+1.5), L(+3.0), DV(-5.0). In the Group II animals, the tip of the guide cannula was lowered to DV(-6.0) from the skull top followed by raising it to DV(-5.0) to make an injection "pocket." It was hypothesized that creating a pocket for the cells to settle into during injection would minimize the spread of tumor cells up the cannula tract. The guide cannula was held in place by cranioplastic cement and cemented to four 6-32-in. stainless steel screws mounted to the cranium. The guide cannula was capped with a dummy stylet that extended 1.0 mm beyond the tip of the guide cannula in order to insure that the guide cannula was clear.

Injecting the Cells

The cells were maintained in suspension by constant agitation and the suspension was injected using a 50-μl syringe. Attached to the syringe was a 5.0-cm piece of polyethylene (PE-20) tubing and a No. 28 stainless steel injection cannula. The cell concentration was rechecked just prior to implantation. The syringe assembly was mounted on a Harvard syringe pump calibrated to deliver 1 μl over 3 minutes. The injection cannula was inserted into the guide cannula. Either the cell suspension or the medium was injected over a period of 10 minutes for Group I and 3 minutes for Group II. In Group I, three sets of five rats each were injected with either 1.0 × 10⁶ C6 cells in 10 μl phosphate-buffered saline (PBS)-glucose medium, 1.0 × 10⁶ Walker 256 cells in 10 μl PBS-glucose medium, or 10 μl PBS-glucose medium (control rats). In Group II, three sets of five rats each were injected with 1.0 × 10⁶ C6 cells in 1 μl PBS-glucose medium, 1 × 10⁵ Walker 256 cells in 1 μl PBS-glucose medium, or 1 μl PBS-glucose medium (control rats). After injection, the injectors remained inside the guide cannula for 5 minutes to allow the injected cell suspension or medium to come to equilibrium inside the brain. The injector was then removed and the dummy cannula was inserted and secured.

Tissue Collection and Processing

The animals were sacrificed 2 weeks after implantation. They were deeply anesthetized with intraperitoneal pentobarbital (50 mg/kg). Group I animals were perfused with normal saline followed by a mixture of formalin, ethanol, and acetic acid (FEA), and Group II animals were perfused with normal saline followed by Bouin’s solution (15 U saturated picric acid, 5 U formaldehyde solution, 1 U glacial acetic acid). The perfusion medium was changed from FEA to Bouin’s solution since the latter is a better fixative for immunocytochemical staining of certain neurochemicals.

The brains were dehydrated and embedded in paraffin. Serial coronal sections of 14 μm thickness were cut throughout the forebrain. Four sections were placed on each slide, and every 10th slide was stained for cresyl violet. In four Group I C6 animals, select slides were stained for glial fibrillary acidic protein (GFAP).

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* Male Wistar rats obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts.
† Tumor cells initially obtained from the American Type Culture Collection, Rockville, Maryland.
‡ Guide cannulas manufactured by Plastic Products, Roanoke, Virginia.
§ Cranioplastic cement and stainless steel screws manufactured by Plastic Products, Roanoke, Virginia.
¶ Hernocytometer manufactured by American Optical, Buffalo, New York.
* Syringe pump obtained from Harvard Apparatus, Millis, Massachusetts.

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**Morphometric Analysis**

First, anterior-posterior (A-P) coordinates for each slide stained with cresyl violet and GFAP were determined using a stereotactic rat atlas by an observer who was blind to the origin of each slide. Second, following these coordinate determinations, morphometric analysis was done using a Loats RAS-1000 system and the tumor and brain areas were determined. Within Groups I and II, the mean tumor area ± standard error of the mean was calculated for each of the three subgroups (C6, W256, and culture medium, with five animals per group) at designated A-P coordinates from +5.7 to −2.3 (from the bregma) at 1-mm intervals, and slices were assigned to these A-P 1-mm serial coordinates by estimating anterior or posterior by ±0.5 mm.

**Tumor Production**

In Group I animals, injection of $10^6$ C6 and $10^6$ W256 cells resulted in tumor being produced in all five rats of both groups. Similarly, in Group II animals, $10^5$ C6 and $10^5$ W256 cells resulted in tumor in all five rats of both groups.

**Morphology**

There was no invasion of the fornix, nucleus accumbens, or hippocampus, nor evidence of hydrocephalus for either of the groups. Representative sections are presented in Fig. 1 and described below.

*Group I.* A coronal section taken through the middle

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**Fig. 1.** Pseudocolor digitized images of coronal sections taken through the largest diameter of the C6 and W256 gliomas stained with cresyl violet at a similar anterior-posterior (AP) coordinate (AP(+0.7)) for Group I, 10 μl volume, and AP(+1.7) for Group II, 1 μl volume. *Upper Pair:* Group I brains injected in the right caudate nucleus with $10^6$ (10 μl) C6 cells (left) or W256 cells (right). The C6 tumor extends to the cortex, and there are some cystic areas in the center of the tumor. The W256 tumor appears round with distinct borders, and small areas of necrosis are noted in the center of the tumor. *Lower Pair:* Group II brains injected with $10^5$ (1 μl) C6 cells (left) or W256 cells (right). The 10-fold decrease in injected cell bulk yielded significantly smaller tumors which are completely contained within the caudate nucleus. The C6 tumor ($10^5$ cells) is much smaller compared to the C6 tumor produced by injecting $10^6$ cells. The W256 tumors were small punctate tumors that were located completely within the right caudate nucleus. Values assigned to AP coordinate intervals are ± 0.5 mm and do not necessarily refer to absolute coordinates in the stereotaxic atlas.
(largest diameter of tumor mass along the A-P axis of all sections examined) of a representative C6 tumor
(AP(+0.7), 10^6 C6 cells) is shown in Fig. 1 upper left. Gross inspection of the cresyl violet-stained
slides revealed that all of the tumors were unifocal masses originating in the right caudate nucleus. Four rats had
tumors that extended to the area of the frontal cortex immediately above the corpus callosum. One rat had a
tumor with a large enough mass to distort the right lateral ventricle. In four rats, C6 cells were found in the
ventricular system and subependymal area and, in four, the cells grew through the corpus callosum. Four of the
five C6 tumors had a cystic component and two had central regions of necrosis.

Figure 2 compares two rats implanted with C6 tumors (both 10^6 cells) at various A-P coordinates showing the extension of these tumors in the brain. The reliability of the model is indicated by the similarity in the distribution of the C6 tumor in these two rats and in others described below at defined A-P coordinates ranging from the most anterior to the most posterior.

Figure 1 upper right shows a coronal section taken through the middle of a representative W256 tumor at the same A-P coordinate (AP(+0.7), 10^6 W256 cells).

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**Fig. 2.** Pseudocolor digitized images of serial coronal sections of two rats in Group I (left and right), both injected with C6 gliomas stained with cresyl violet. Each rat was injected with 10^6 cultured C6 glioma cells in a volume of 10 μl in the right caudate nucleus. The projection of tumor is shown from the most anterior (upper) to the most posterior (lower) extension with the middle section depicting the largest tumor area. Note the similarity of the tumor in these randomly selected subjects. In all rats studied, tumor was found in the right caudate nucleus but not in the nucleus accumbens, fornix, or hippocampus.
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Three of the five W256 tumors were subcortical, with two completely enclosed within the caudate nucleus. Two tumors produced distortion of the right lateral ventricle. No W256 cells were identified in the ventricular system or in the subependymal area. None of the tumors had a cystic component but three showed at least one area of central necrosis. None of the W256 tumors extended to the top surface of the cortex.

Group II. A coronal section taken through the middle of a C6 tumor (AP(+1.7), 105 C6 cells) is shown in Fig. 1 lower left. The injected bulk of tumor mass was reduced by 10-fold in comparison with that received by the Group I C6 cell recipients (compare with Fig. 1 upper left). Each of the tumors was subcortical and completely enclosed within the right caudate nucleus; there was no distortion of the right lateral ventricle or gross invasion of the corpus callosum. In one rat, C6 cells were found in the ventricular system and subependymal area. Two of the five C6 tumors in Group II showed cystic components and two had areas of central necrosis.

Figure 1 lower right shows a coronal section taken through the middle of a W256 tumor (AP(+1.7), 105 W256 cells) where the injected bulk of tumor mass was reduced by 10-fold in comparison with that illustrated in Fig. 1 upper right. All of the tumors were subcortical and completely enclosed within the right caudate nucleus. Two of the five tumors produced a mass effect that distorted the right lateral ventricle. There was no invasion of the ventricular system or corpus callosum.

One tumor had a cystic component and none had areas of necrosis.

Morphometric Analysis

Group I. Figure 3 upper pair shows the brain-tumor profiles for C6 and W256 tumors at specific A-P coordinates (105 cells injected in 10 μl PBS-glucose medium). The largest cross-sectional tumor area was centered approximately at the injection site, A(+1.5). The C6 tumors extended anteriorly 1.0 mm further than W256 tumors but posterior extension was the same for the two cell types (Fig. 3 upper pair). The mean tumor areas for C6 tumors were (in sq mm): A(+4.7) 0.4 ± 0.2; A(+3.7) 3.5 ± 1.1; A(+2.7) 5.7 ± 1.7; A(+1.7) 9.5 ± 2.3; A(+0.7) 7.5 ± 3.2; A(0.3) 3.7 ± 2.9; and A(-1.7) 0.3 ± 0.3. The mean tumor areas for W256 tumors were (in sq mm): A(+3.7) 0.4 ± 0.4; A(+2.7) 7.2 ± 3.9; A(+1.7) 9.9 ± 2.1; A(+0.7) 7.7 ± 2.4; A(-0.3) 2.8 ± 1.7; and A(-1.3) 0.2 ± 0.2.

Group II. Figure 3 lower pair shows the tumor area profiles for C6 and W256 tumors at specific A-P coordinates (105 cells injected in 1 μl PBS-glucose medium). Reducing the injected tumor bulk from 105 to 104 cells resulted in a decreased spread along the A-P axis. The mean tumor areas for C6 tumors were (in sq mm): A(+4.7) 0.8 ± 0.4; A(+0.7) 0.7 ± 0.2; and A(0.3) 0.2 ± 0.2. The mean tumor areas for W256 tumors were (in sq mm): A(+3.7) 0.2 ± 0.2; A(+2.7) 1.2 ± 1.2; A(+1.7) 4.0 ± 2.1; A(+0.7) 7.0 ± 1.4; and A(0.3) 2.2 ± 1.2.

Fig. 3. Graphs showing mean cross-sectional areas of C6 and W256 tumors at successive 1-mm antero-posterior (AP) coordinates. Tumor area was determined using a Loats RAS-1000 system. There was low variance between the five animals in each treatment set and each cell type, indicating reproducibility. Data are shown for Group I animals injected with 105 C6 cells (upper left) and with 105 W256 cells (upper right) in 10 μl phosphate-buffered saline (PBS)-glucose medium and for Group II animals injected with 104 C6 cells (lower left) and with 104 W256 cells (lower right) in 1 μl PBS-glucose medium. The bregma is at AP (0).
Histological Findings

C6 Tumors. Staining of the C6 cells from Group I rats for GFAP showed that the C6 cells had large nuclei and possessed abundant cytoplasm, and there were many thick, short processes extending in all directions. The sections showed crowded C6 cells overlapping in the center of the tumor with occasional areas of cyst formation and necrosis. Surrounding the tumor were individual C6 cells migrating into the brain parenchyma. The C6 cells were found in the corpus callosum, the subependymal area, and along the blood vessels. There was no evidence of secondary tumor formation. Morphometric analysis of tumor areas at the same coordinate (largest diameter), stained with cresyl violet or GFAP, in four rats injected with C6 cells in 10 μl failed to indicate a significant difference in the tumor areas shown by these two staining techniques (cresyl violet 10.76 ± 7.81, GFAP 10.57 ± 8.08; t(3) = 0.75, and p > 0.10).

W256 Tumors. The W256 cells of Group I rats in sections stained with cresyl violet exhibited round cells with large nuclei. The cells were arranged in a whorled pattern at the center of the tumor. There was rare cyst formation and occasional tumor necrosis; no evidence was found of cell migration away from the tumor mass or of secondary tumor formation.

Discussion

Highly reproducible tumors were produced in rats using a stereotactic procedure involving implantation of a permanent stainless steel cannula and injection of cultured cells into the caudate nucleus. All masses were well localized and originated at the right caudate nucleus, and no secondary masses were produced. There was no extension of tumor up the injection track or around the stainless steel cannula as occurs with injection via a syringe. 4,5,6 By comparison, seeding of tumor cells along the line of injection is almost always the case with free-hand systems. The number of cells used in both groups (106 and 107) was sufficient to produce tumor for both C6 and W256 cell types in 100% of cases. Reduction in cell number from 106 to 105 cells and injection volume from 10 to 1 μl greatly decreased C6 and W256 tumor magnitude and the distribution along the A-P axis. The relatively greater tumor mass produced by the 1-μl (106) injection of W256 tumors versus C6 cells is not explained. This may be an artifact of the small sample size combined with the decreased reliability of the smaller (1 μl) tumor volume.

With staining for GFAP it was possible to morphologically distinguish C6 cells from normal astrocytes in the host brain. 7 As noted by Bernstein, et al., 8 C6 cells had larger cell bodies, larger nuclei, and less extensive but thicker and shorter cell processes than normal astrocytes. In rats injected with 106 cells (10 μl), individual C6 cells were found in the corpus callosum, in the subependymal area, and in the surrounding blood vessels. It has been shown that C6 cells migrate along such structures. 9 By comparison, the injected W256 cells formed a discrete round mass centered on the injection site. The pattern of growth of the tumor mass was similar to the tumors produced by intracarotid injection of W256 cells 10 except that there were no secondary areas of tumor seeding. The tumor borders were well circumscribed.

Morphometric analysis of C6 tumors with GFAP and cresyl violet staining yielded similar tumor areas. Cresyl violet (a nuclear stain) stains cell bodies while GFAP specifically stains GFAP in the cell processes of the C6 cells and host brain astrocytes. Our results indicate that cresyl violet is sufficient to achieve morphometric analysis of C6 gliomas and W256 tumor areas.

An advantage of our system is that it is not limited to any particular tumor or brain site. The reproducibility of the model makes it useful for investigating the effects of drugs on brain-tumor growth and inhibition in vivo. Indeed, the presence of a permanent cannula at the center of the tumor offers a unique opportunity to examine the effects of focal application of chemotherapeutic agents directly into the tumor mass. Such an approach would bypass the blood-brain barrier. This model may be useful as a standard for various types of brain-tumor studies.

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Address reprint requests to: Barbara H. Herman, Ph.D., Medications Development Division, National Institute on Drug Abuse, National Institutes of Health, 3600 Fisher's Lane, Room 11A-55, Rockville, Maryland 20857.