The development of an intracerebral glioma model for brain tumor chemotherapy

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The authors describe a brain tumor model for chemotherapy studies. The tumor is an intracerebral ependymoblastoma that kills the host in a short time (median survival, 27.5 days) and yields consistent, uniform survival curves. A suspension of tumor cells is injected into the right frontal lobe of the mouse by means of a stereotaxic frame, and produces a highly invasive, almost entirely intracranial brain tumor. The use of mice permits extensive chemotherapeutic trials for brain tumors at low cost. It is felt that this model will prove to be very useful for studies of brain tumor chemotherapy.

KEY WORDS • experimental brain tumor • chemotherapy

Chemotherapy has not been a very effective method of treating most types of malignant brain tumors. A brain tumor model in experimental animals that closely reflects the clinical situation would be useful for testing chemotherapeutic agents for possible clinical use.

The model we are reporting is that of a glial tumor transplanted intracerebrally into mice where it grows rapidly to produce death in greater than 97% of the animals within 60 days. The method of implantation is speedy and lends itself to large-scale chemotherapeutic studies.

Materials and Methods

Female mice (No. C57BL/6J) weighing 16 to 18 gm were obtained from Jackson Laboratory, Bar Harbor, Maine. We used a mouse ependymoblastoma which has been maintained in our laboratory for the past 9 years by serial subcutaneous transplantation every 2 weeks of tumor fragments into the lower abdominal wall. The tumor was originally induced by Zimmerman and Arnold in mouse brain with intracerebrally-implanted methylcholanthrene.

Preparation of Tumor Cell Suspension for Transplantation

Two-week-old subcutaneous tumors are harvested from a sufficient number of mice to yield 2 gm of tumor tissue. This material is then minced with a scalpel and washed with calcium-magnesium (Ca-Mg)-free phosphate-buffered saline and placed in 50 ml centrifuge tubes. Following centrifugation at 1000 rpm in a Sorvall GLC-1* for

*Sorvall GLC-1 manufactured by Ivan Sorvall Inc., Norwalk, Connecticut 06852.
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7 min, the supernatant is discarded and the tumor mince is then added to 30 ml of a trypsinization medium containing 0.0025% trypsin and 0.1% EDTA dissolved in the Ca-Mg-free buffered saline. The tumor mince is then gently stirred in this medium at 37°C for 10 min. The suspension is allowed to settle for 1 min after which 15 ml of the supernatant containing suspended tumor cells is drawn off and placed in the refrigerator. Fifteen ml of fresh trypsin-EDTA solution is then added to the trypsinization medium, and gentle stirring restarted. The whole process is repeated three more times, following which the pooled suspensions are filtered through a stainless steel screen to remove any large particles that might later block the 30-gauge needle used for injection. The filtrate is centrifuged at 1000 rpm for 10 min, the pellet is resuspended in the Ca-Mg-free phosphate-buffered saline containing 0.1% EDTA, and then recentrifuged. The final pellet is resuspended in 0.4 ml of Ca-Mg-free phosphate-buffered saline with 0.1% EDTA and a cell count made using a white blood cell (WBC) counting chamber. The final tumor cell suspension is adjusted to yield 1.0 x 10^8 cells per ml, and includes 50 units of penicillin and 50 μg of streptomycin per ml.

The suspension is loaded into a silicone lubricated 2.5 ml Luerlok syringe* fitted with a 30-gauge needle. The syringe and needle are attached to a Gilmont micrometer assembly† by means of a metal adaptor. Each mouse receives an intracerebral injection of 300,000 cells in a volume of 3 μl.

Preparation of Tumor Fragments for Transplantation

Two-week-old subcutaneous tumors from three mice are placed in a saline-moistened Petri dish kept on ice. Non-hemorrhagic tumor is selected and cut into slices on a Stadie-Riggs microtome.‡ Tumor slices are

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* Luerlok syringe manufactured by Becton, Dickinson & Company, Canada Ltd., Clarkson, Ontario.
† Gilmont micrometer manufactured by Roger Gilmont Company, 161 Great Neck Road, Great Neck, New York.

The essential part of the stereotaxic frame is an acrylic block 3 cm high x 2.5 cm wide x 1.5 cm deep with a conical hole through its depth and with the axis of the cone parallel to the horizontal plane. The block was shaped (cast) around the head of a mouse, and the hole (Fig. 1) was fashioned to allow the nose of a 16 to 19 gm mouse to project 3 to 5 mm beyond the acrylic block. To firmly secure the mouse's head in the block, a wire loop on the end of a sliding bar is placed around the mouse's upper incisor teeth and the bar is pulled tight and
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then clamped. The tumor cell suspension or fragments are injected through a second hole in the block placed to allow injection into the mouse’s right frontal lobe. This hole is far enough lateral to avoid intraventricular injection, but sufficiently medial to avoid subdural injection.

A short, beveled 23-gauge needle is inserted through the vertical hole in the acrylic block far enough to penetrate the mouse calvarium. A 30-gauge needle mounted on the end of the micrometer syringe assembly is then inserted through the lumen of the 23-gauge needle and projected beyond its tip a depth of 3 mm into the right frontal lobe. A turn of the micrometer delivers 3 μl of cell suspension.

After the needle is withdrawn from the animal, the micrometer is turned to be sure that the needle has not blocked. If the needle is found to be blocked, it is replaced and the last mouse injected with it is excluded from the experiment. After each injection, the 23-gauge needle is flushed with sterile water and the 30-gauge needle wiped with gauze soaked with 70% alcohol. The suspension-laden syringe is agitated between injections to prevent settling of the suspended cells and to ensure consistency in the number of tumor cells injected. The whole injection process is fast, and 80 mice can be injected in less than 1 hour.

For implanting tumor fragments the same basic stereotaxic frame is used but with some minor modifications. The vertical hole in the acrylic frame is enlarged to accommodate an 18-gauge trocar. With the mouse in place, the trocar is inserted through the hole just far enough to penetrate the calvarium and is then removed. The tumor-bearing trocar is then inserted through the hole and calvarium and penetrates the right frontal lobe a distance of 2 mm. The stylet is advanced and projects 1 mm beyond the trocar and deposits the tumor fragment in the brain.

Experimental Protocol

Tumor suspensions were given to eight groups of 25 mice each. Three groups received the same suspension and the other five groups received suspensions prepared at different times. Tumor fragments were given to one group of 30 mice. All long-term survivors (over 60 days) were sacrificed and the brains examined histologically. Additional animals received tumor suspension or tumor fragments and were examined histologically immediately after implantation and at 4 days. Representative samples of animals dying of brain tumor were also examined histologically.

Results

The intracerebral tumor implantation procedure was well tolerated by the mice. Only two of the 200 mice receiving the intracerebral tumor suspensions died during or shortly after the injection; death was due to asphyxiation from being kept in the frame for longer than 30 sec. In the group receiving solid tumor fragments there were no immediate deaths, but two mice died 2 hrs after injection. Autopsy showed a large intracerebral hematoma in both, and this was felt to be the cause of the delayed death.

Almost all the mice were rendered unconscious as a result of being clamped in the frame, and the unconscious period lasted about 10 sec, after which the mice walked about the cages normally. From then until about the seventh day the mice appeared normal, after which some began to change in behavior and appearance. The affected mice lost weight, became less active, had a tendency to walk high on their hindlimbs, and showed gradual enlargement of the head. A small proportion of the mice that received tumor suspension had a small extracranial tumor nodule at the site of injection. In contrast, all the mice that received solid tumor fragments had a large subcutaneous extracranial tumor mass that appeared early following implantation and often produced ulceration of the overlying scalp.

Table 1 shows the median day of death and number of long-term survivors in the eight groups of mice that received tumor suspension and the one group that received solid tumor fragments. The median day of death for the eight suspension groups varied from 21 to 31 days, with an average of 27.5 days. In contrast, the median day of death
TABLE 1

Median day of death and number of long-term survivors after intracerebral tumor implantation

<table>
<thead>
<tr>
<th>Implantation</th>
<th>No. of Mice</th>
<th>Median Day of Death</th>
<th>Long-term Survivors (more than 60 days)</th>
</tr>
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<tbody>
<tr>
<td>tumor suspension:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>group I</td>
<td>25</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>28</td>
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<td>3</td>
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<td>total</td>
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<td></td>
<td>6</td>
</tr>
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<td>average</td>
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<tr>
<td>solid tumor fragments</td>
<td>30</td>
<td>37</td>
<td>0</td>
</tr>
</tbody>
</table>

For the tumor fragment group was 37 days.

Of the 200 mice that had received the tumor suspension, there were six long-term survivors (>60 days), with two groups having one each, and two groups having two each. Histological examination of these six survivors showed extensive intracerebral tumor growth in one, a few tumor cells in the meninges of two others, and no evidence of tumor in the remaining three. Figure 2 shows the survival curve of one group of mice that had received the tumor suspension. The first death occurred on the 8th day, the last death on the 34th day; the median day of death was the 24th. There were no long-term survivors in this group of 25 mice.

Histological examination of mice killed immediately after the injection of tumor suspension showed surprisingly little disruption of cerebral tissue. When the brain was removed from the cranial cavity the site of injection was visible from the right lateral surface as a thin dark line. However, there was no evidence of cortical disruption on the inferior or lateral surfaces of the brain. At superficial levels along the needle tract there was only a small amount of hemorrhage while at deeper levels the tract was filled with tumor cells and red blood cells. Tumor cells were also seen lying along blood vessels up to 0.5 mm away from the injection site.

Four days after implantation of the tumor suspension there was active tumor proliferation along the needle tract and infiltration of surrounding brain (Fig. 3). There was also subarachnoid tumor growth that was thickest at the site of injection. Mice dying early (up to approximately the 25th day after implantation of tumor suspension) showed expansion of the right side of the head, and usually no evidence of extracranial tumor at this time. Microscopic study (Fig. 4) showed extensive growth of tumor in the cerebral cortex with widespread infiltration of tumor deep into white matter and frequent growth across the midline into the left hemisphere. In all the mice examined there was extension of the tumor extracerebrally into the subarachnoid and subdural spaces.

When examined 25 days following implantation of tumor suspension the skulls
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were usually bulbous with many small adherent lumps of tumor found along the suture lines. There was massive replacement of cerebral tissue by tumor (Fig. 5) which varied from growth throughout the entire right hemisphere to almost total replacement of both hemispheres by tumor. By this time there was always some growth of tumor through the site of implantation, the skull was infiltrated by tumor, and the sutures were split allowing tumor to grow extracranially. However, the amount of extracranial tumor as compared with intracerebral tumor was always very small.

The mice in which tumor fragments had been implanted all developed a large extracranial tumor mass within 25 days of implantation. Microscopic examination showed this mass to be growing primarily through the skull defect created by the trocar at the time of implantation (Fig. 6). The intracranial tumor showed the same subdural, subarachnoid, and intracerebral growth pattern as that found with the suspension technique. At the later times the huge extracranial tumor mass comprised one-third to one-half the total amount of tumor present.

Discussion

The ideal brain tumor model for chemotherapy experiments should include the following characteristics: 1) the tumor should be of glial origin; 2) the tumor should grow intracerebrally without extracranial components; 3) tumor cells should infiltrate the surrounding brain as do spontaneous glial tumors and not merely grow as an expansile mass; 4) the period of time from implantation to death should be short and uniform; 5) a small animal is preferable so that sufficiently large numbers could be treated to yield statistically valid results at low cost. Although several brain tumor models have been developed none is ideal for chemotherapeutic experiments. For example, some models consist of intracranial implants of nonglial tumors,7-9 such as L1210 leukemia, uterine epithelioma (Ts Guérin), Sarcoma 180, or Ehrlich carcinoma; the intracerebral growth pattern of these tumors is quite different from that of an intracerebral glioma.

Confinement of the experimental brain tumor to the intracranial location without extracranial extension is considered important in chemotherapeutic studies because of the known differences in the vasculature and the distribution of drugs in tumors grown intracerebrally compared with those grown subcutaneously. For example, Torack11 demonstrated the presence of endothelial pores in a subcutaneous ependymoblastoma but not in the same tumor growing intracerebrally. These pores are considered an important route for the transport of agents from the bloodstream into tumors. They may account for the differences in the 14C-inulin space between subcutaneous and intracerebral implants of the same mouse ependymoblastoma found by Ausman and Levin.1 These same authors4 found the uptake of 3H-methotrexate in subcutaneous implants of mouse ependymoblastoma to be approximately 50% greater than in intracerebral implants of the same tumor. Therefore, in a
brain tumor model for chemotherapeutic studies it is considered important that the glial tumor be wholly dependent on intracranial vessels since a large extracranial component would obtain its blood supply from noncerebral blood vessels. To stimulate the conditions present in patients with malignant gliomas, drugs should reach the experimental brain tumor only via intracranial vessels and not via an extracranial route.

Infiltration of normal brain is one of the characteristics of spontaneous malignant glial tumors and should also be one of the characteristics of a glial tumor model. Although the blood-brain barrier is altered within brain tumors and permits entry of chemotherapeutic agents, tumor cells deeply invading normal brain will be more shielded from chemotherapeutic agents by the blood-brain barrier in the adjacent normal brain. This has been demonstrated autoradiographically with $^3$H-methotrexate. When the drug was given intravenously there was good uptake by the neoplastic cells in the main intracerebral tumor mass, but scant uptake by tumor cells infiltrating deeply into the surrounding brain. Rosso, et al., implanted Sarcoma 180 and Ehrlich carcinoma in three different sites, intracerebral, intraperitoneal, and subcutaneous, and treated the animals with cyclophosphamide and sarcolysin. The subcutaneous and intraperitoneal tumors responded to treatment but the intracerebral tumors did not. By analogy to the findings with methotrexate, the deeply infiltrating tumor cells were probably not reached by these chemotherapeutic agents, and hence the animals failed to respond to them. In addition, these experiments indicate the necessity for using an intracerebral tumor for brain tumor chemotherapy studies.

Ideally in a brain tumor model for chemotherapy, the tumors should cause death within a short time interval, less than 60 days, to obtain results within a reasonable period. Moreover, the number of long-
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Fig. 5. Photomicrograph of anterior view of decalcified coronal section of brain in a mouse 30 days after injection of suspension. The darker areas are tumor that has replaced most of the right hemisphere and has compressed the left hemisphere. Tumor can be seen invading the skull (pale line, arrow) and growing through split suture lines (arrowhead). There is a ribbon of tumor outside the skull at the top left. H & E, X 23.

term survivors in untreated groups must be small so that any increased survival in treatment groups can be attributed to drug action and not to chance or technical error. A small animal model keeps the cost of the experiment low, and permits statistically significant samples to be tested.

There are two brain tumor models used for chemotherapy that fulfill most of these criteria. Wilson, et al., used a stereotaxic frame to implant tumors into rat brain and achieved a wholly intracerebral tumor. However, rats are more expensive to purchase and maintain than mice, and Wilson’s implantation procedure required many more manipulative steps, such as making a skin incision, drilling a burr hole, inserting a hollow stainless steel screw, and plugging the screw with bone wax after tumor implantation. This elaborate procedure was designed to prevent extracranial tumor growth. In our model there was no growth through the implantation site in mice dying within 17 days of implantation. However, all mice dying after 17 days showed some growth of tumor through the site of implantation. The amount of extracranial growth at this site was always directly proportional to the amount of extracranial tumor growing through split cranial sutures, and in all instances the total extracranial tumor growth was very small compared to the huge intracranial tumor mass (Fig. 5). Ausman, et al., developed a brain tumor model for chemotherapy using the Zimmerman ependymoblastoma in mice. They used free-hand placement of a solid fragment of tumor for intracerebral implantation, and over 90% of their animals developed extracranial tumor nodules. Although their model is a good one, it is felt that the stereotaxic frame used in the
Fig. 6. Photomicrograph of posterior view of decalcified coronal section of brain in a mouse 35 days after implantation of solid tumor fragment. The skull (pale line, arrow) is discontinuous under the large extracranial tumor mass at the site of implantation. The extracranial tumor (arrowhead) is almost equal in size to the intracranial tumor (crossed arrow). H & E, X 23.

Present experiments provides greater control of tumor placement, and produces fewer extracranial subcutaneous tumor nodules. In addition, free-hand placement, as Ausman, et al., pointed out, could result in intraventricular tumor growth, a pattern of growth we particularly wanted to avoid since it has been shown that intraventricular ependymoblastoma did not take up any intravenously administered methotrexate.11

We feel that our model combines the best features of the previous models in that a glial tumor is used and is transplanted stereotaxically using a technique which is fast, accurate, safe, and results in a uniform growth with few long-term survivors. The stereotaxic frame was used for both the placement of the tumor fragments and the tumor suspension. The suspension technique is preferred because it does not result in large extracranial subcutaneous tumor nodules. The number of long-term survivors in this group of experiments was 3%, and all of these occurred in the first four groups of animals receiving transplants. The last four groups of animals had no long-term survivors, so that with experience it is possible to achieve a very low rate of long-term survivors.

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References

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