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A METHOD TO ACCURATELY INJECT TUMOR CELLS INTO THE CAUDATE/PUTAMEN NUCLEI OF THE MOUSE BRAIN

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OBJECTIVE: To improve currently used techniques to implant tumor cells into the parenchyma of the mouse brain.
MATERIALS AND METHODS: The stereotactic injection of 0.5 to 5 μl of indigo carmine over 5 to 40 minutes into the caudate/putamen nuclei of the mouse was done followed by sacrifice and examination of the brain injection site.
1 μl containing 10^5 U87MG glioma cells were stereotactically implanted into the caudate/putamen nuclei over 20 minutes. The animals were sacrificed from one hour to 63 days after implantation and the brain examined and tumor size measured.
RESULTS: An injection of 1 μl of indigo carmine over 20 minutes produced a spherical deposit of dye within the caudate/putamen nuclei. Larger volumes of indigo carmine or shorter injection times resulted in dye spreading along the injection tract or into the ventricles or subarachnoid space.
Using the results of the dye studies, the same parameters were used to successfully inject and confine the glioma cells to the caudate/putamen nuclei in 30 of 32 mice. No tumor was found in 2 animals and appears to be explained by obstruction of the injection cannula.
The tumor cells appeared viable an hour after injection. However by day three, considerable necrosis of tumor cells were noted, the effects of which resolved by day five. On day six, the injection site was comparable to that at one hour.
In the early phase, until the fifth week, tumor volume doubling time was ten days while afterward it was only five days.
CONCLUSION: The technique described allows the highly accurate and reproducible introduction of a given number of cells into a specific area of the mouse brain. This should reduce the intragroup variability, be it control or therapeutic, allowing better assessment of outcome with fewer number of mice.

Key words : brain tumor model, xenograft, nude mice

INTRODUCTION

The therapeutic response of various agents to brain tumor cell lines is frequently tested in immunodeficient mice following subcutaneous or intracerebral injection. The subcutaneous injection allows one to evaluate the response to treatment by simply measuring tumor size with time and constructing a growth curve [12, 15, 17, 20-22, 27]. This technique, however, does not well replicate the intracranial situation wherein the blood-brain barrier can have significant influence on tumor growth and its response to therapeutic agents [18]. The growth of intracranial tumors is often evaluated by

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sacrificing the animal at time intervals and measuring the size of the tumor, a technique that requires the use of a large number of animals and adds to the cost. A more frequently used method is to record the time between injection of tumor cells and the death of the animal; comparing the control to the treated group. The less intragroup variability the fewer the number of animals are needed to establish the presence or absence of significant therapeutic effect.

The experimental intracranial injection techniques previously reported [1, 10, 11, 13, 16, 17, 19-21, 23, 25, 26] do not necessarily or accurately assess the following; the number of cells injected, their viability after injection, the degree of anatomical disruption of the injection site, whether the tumor cells are confined to the parenchyma or have been injected into the ventricles, subarachnoid space, or even subcutaneous tissues, all factors that will influence tumor growth and lead to variability in the time between tumor injection and the animal's death.

In this report, we describe a technique to accurately implant a precise number of tumor cells within a given location in the mouse brain, greatly reducing the variability of the experimental setting.

MATERIALS AND METHODS

Cell Line: The human brain tumor cell line U87 MG (glioblastoma) was purchased from ATCC, Manassas, VA and grown in PRMI with 10 % fetal bovine serum in 5 % CO_2 at 37 ˚C. Cells for intracerebral injection were obtained by trypsinization from sub confluent cultures, washed twice with PBS, and resuspended in serum-free RPMI 1640 medium at 10^8/ml.

Tumor Implantation: All animal studies were done according to the NIH guidelines and approved by the institutional animal care committee. Female Balb/c nu/nu mice (Harlan Sprague Dalley, St. Louis, MO), 4 to 6 weeks old, were anesthetized with 200 μl of a mixture of Ketamine and Xylazine by intra-peritoneal injection at kept at 37 ˚C using a warming pad. The head was fixed in the stereotactic frame (Kopf Instruments, Sunland, CA) using ear bars specially shaved for mice. A 1 cm midline scalp incision was made and the bregma set in the same plane as the lambda by adjusting the stereotactic frame. A small hole was made into the skull with a #33 G drill bit 0.5 mm anterior and 2 mm lateral to the bregma. A 33-gauge injection cannula (Plastic One, Inc.) was then inserted at 3.3 mm depth from the skull surface and connected to the injection systems shown in Fig. 1. This system was developed to allow very slow injection rate (0.05 μl/min) of infusate in order that the pressure created at the injection site was minimized. This brain micro-injection system was originally developed by Cserr *et al.* [3, 4, 28] for extra-cellular fluid clearance studies in the brain. The injection system is composed of

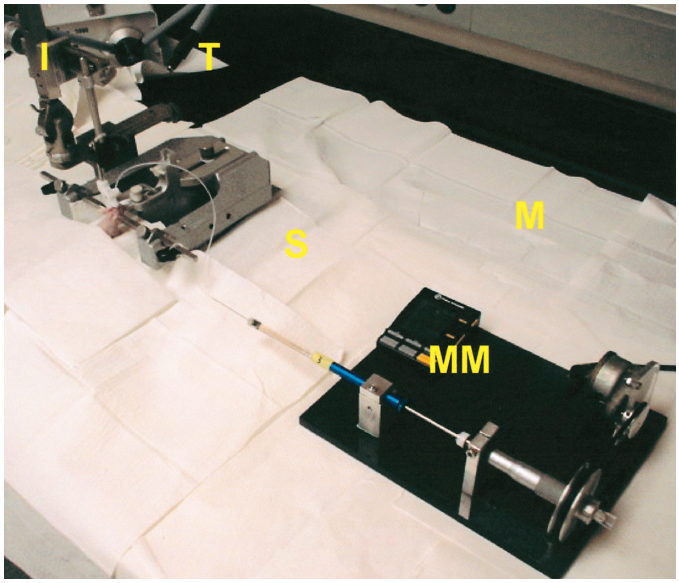

Fig. 1 Injection system for tumor implantation.
S: airtight 10 μl syringe
MM: micrometer
M: motor
T: 28G Teflon tubing
I: 33G injection cannula in device

an injection cannula, connecting tube (28G Teflon tubing), micro-syringe, micrometer, and stepping motor. The micrometer, connected by a pulley and a belt, is driven by the slow moving stepping motor. The micro-syringe plunger is fitted with air-tight Teflon tip (Hamilton syringe 80428 RN NDL (22s/2"/2) to prevent any leakage around the plunger. The system is non-expandable assuring an accurate injection volume is delivered at a slow speed. The injection cannula was left in place for 5 min after termination of the injection to minimize reflux. The borehole was occluded with bone wax and the incision closed using wound glue.

Pathology

The brains were removed following vascular perfusion with a solution containing PBS and buffered 10 % formalin and then either snap frozen in OCT or fixed in 10 % buffered formalin for paraffin embedding. Samples were sectioned and stained with hematoxylin and eosin (H&E).

Determination of tumor size

Maximal and minimal diameter of the tumor was measured in mm on H&E stained sections and the volume calculated using the following formula: Tumor volume (mm^3) = (max. diameter) × (min. diameter)^2 × 0.5 [21].

RESULTS

Injection volume and speed:

Indigo carmine was injected into the caudate/putamen nuclei of a group of mice to determine the appropriate speed and volume of injection. Injection volumes were 0.5, 1.0, 3.0, and 5.0 μl delivered in 5 to 40 min. There was a spherical area of dye confined to the caudate/putamen nuclei when the injection volume was 0.5 or 1 μl infused over 20 min (Fig. 2A). When the infusion rate was shortened to 5 min, noticeable reflux into the needle track was noted (Fig. 2B). Injection volumes of 3 or 5 μl dye over 40 min resulted in considerable overflow of indigo carmine into the ventricles for either volume and reflux of dye to the brain surface with 5.0 μl (Fig. 2C, D). The optimal injection rate was determined to be 0.05 μl/min. Based upon these results, we used a volume of 1 μl containing 10^5 cells injected for 20 min for the rest of the studies.

Tumor cell injection and tumor growth

U87 glioma cells were suspended at 10^5 cells/μl of serum free 1640 RPMI medium and injected over 20 min. The injection cannula was withdrawn 5 min post inoculation to allow the tumor cells to settle into the caudate/putamen region. The number of tumor cells/μl was determined prior and after injection to verify that the desired number of cells were delivered to the target site.

The animals were killed and the brains examined at various time points after the tumor inoculation starting from one hour after injection to day 63. The injected tumor cells appear viable 1 hr after injection, as shown in Fig. 3A. Considerable central and some peripheral necrosis is seen on day 3 (Fig. 3B) that is nearly resolved by day 5 (Fig. 3C). Tumor growth is observable by day 6 (Fig. 3D).

Injected tumor cells form a sphere confined to the caudate/putamen nuclei with no noticeable reflux along the needle tract on day 1 (Fig. 4A). After 28 days, the tumor has slowly grown, doubling the initial injection

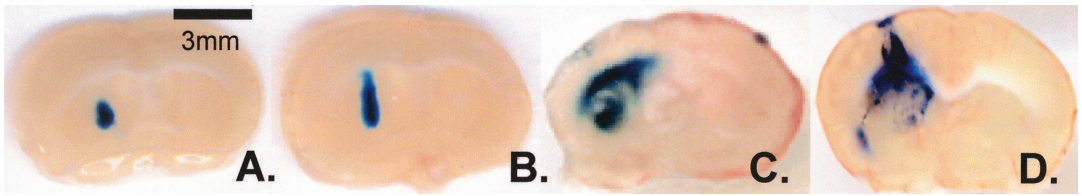

Fig. 2 Dye injection study. Indigo carmine was injected into the right caudate/putamen nuclei at different volumes and injection speeds to determine the optimal parameters.
A: 1 μl/20 min. Spherical area of the dye confined to the caudate/putamen nuclei.
B: 1 μl/5 min. Noticeable reflux of dye along the cannula tract.
C: 3 μl/40 min. Reflux of the dye into the ventricles.
D: 5 μl/40 min. Reflux of dye into the ventricles and subarachnoid space.

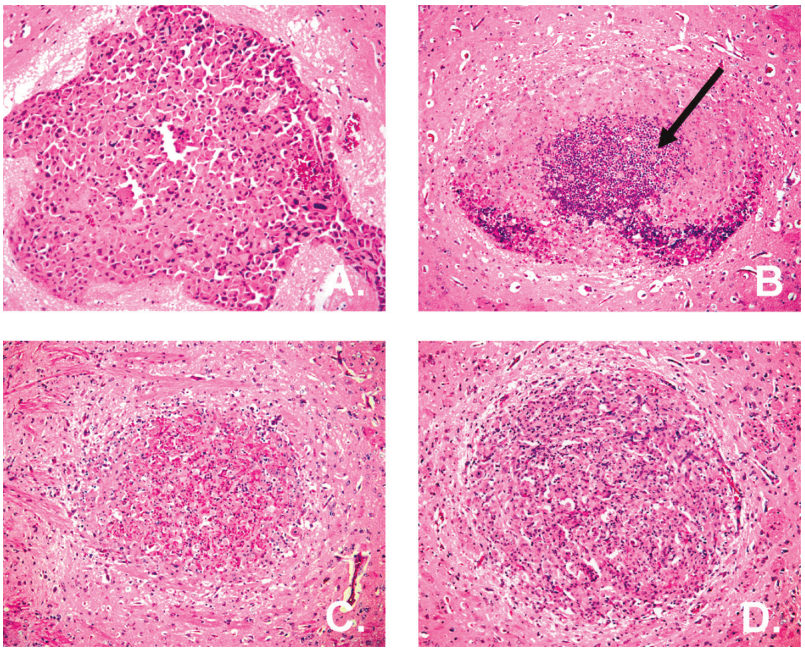

Fig. 3 Histology of the early stages after tumor implantation (H&E staining, magnification 10×)
A: 1 hour. The tumor cells appear to be viable.
B: Day 3. Considerable central (arrow) and some peripheral necrosis is seen.
C: Day 5. Areas of necrosis nearly resolved.
D: Day 6. Tumor growth is noted and comparable to the number of cells seen 1 hour after Injection.

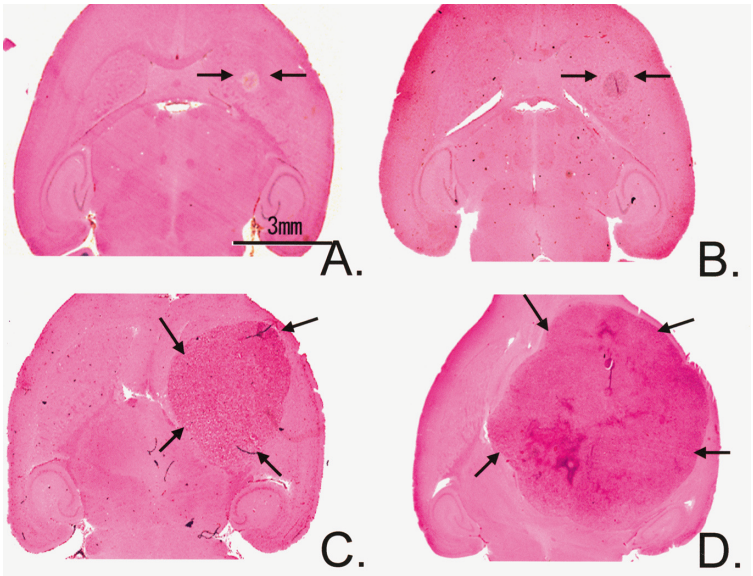

Fig. 4 Growth of tumors following implantation (H&E staining, size bar of 3 mm is shown in A).
A: Day 1. Tumor cells form a sphere confined to the caudate/putamen nuclei with no reflux along the cannula tract.
B: Day 28. The tumor has slowly grown, doubling the initial injection volume.
C: Day 49. The tumor fills more than half the brain hemisphere.
D: Day 56. The tumor fills most of the hemisphere and produces considerable mass with considerable midline shift compromising the opposite hemisphere.

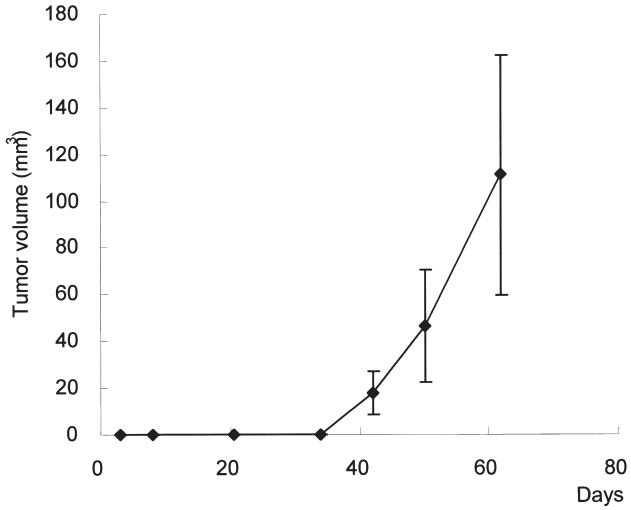

Fig. 5 Intracerebral growth of U 87 10^5 tumor cells injected into the caudate/putamen nuclei of mice. The animals were sacrificed at the times indicated. Tumor volume is given in mm^3.

volume (Fig. 4B). After 49 days, the tumor fills more than half of the hemisphere (Fig. 4C) and by day 56, the hemisphere is almost completely replaced by the tumor producing midline shift with compression of the opposite hemisphere (Fig. 4D).

Tumor growth was initially slow with the volume remaining at 1-2 mm^3 (early phase) until week 5 after which exponential growth occurred (late phase) and all mice were sacrificed by week 9 due to rapid clinical deterioration from tumor progression. The average tumor volume at sacrifice was 110 mm^3 (Fig. 5). Tumor volume doubling times in the early and late phases were approximately 10 and 5 days respectively.

Histologic examination of the brains confirmed successful tumor implantation into the caudate/putamen nuclei in 30/32 animals. In the two animals in whom there was no tumor growth, analysis of the cell count after the injection suggested that the cannula was most likely occluded. No subarachnoid or intraventricular tumor growth was observed in any of the animals and the CSF pathways were open.

DISCUSSION

As mice are inexpensive and readily obtainable, they are one of the most commonly used of animal models. Their small size, however, can be a disadvantage. Using less precise injection techniques and fluid volumes of 3-10 μl, we, as have others, have noted a wide variation in the survival times in both the controlled and therapeutically treated groups of mice [1, 5, 6, 12, 14, 18, 24]. These less precise techniques can result in tumor cells being delivered to wide areas of the brain, ventricles, subarachnoid space, or even extradurally that will have a significant effect on the rate of tumor growth. The ability with high accuracy to deliver a precise number of tumor cells to a specific area of the brain should greatly reduce this variability in either the control or therapeutically treated groups. This should allow the better assessment of various therapeutic agents with fewer mice.

The caudate/putamen nuclei was chosen as the delivery site of tumor cells as it has a volume of 8-9 μl which includes an extracellular space of just over 1 μl. Thus, injection volumes of 2 μl or more will result in tumor cells being widely dispersed into a large area of the parenchyma, ventricle, or subarachnoid space as are dye studies showed when 2-5 μl were injected even though the duration of infusion was up to 40 minutes (Fig. 2). Reducing the injection volume to 1 μl given over 20 minutes uniformly results in a tightly confined spherical area within the caudate/putamen nuclei with no reflux along the cannula tract. The tech-

nique described also avoids an implanted cannula so as not to provoke an inflammatory response and the possible induction of traumatic angiogenesis.

There was observed very little traumatic brain damage due to the cannula insertion or tumor cell infusion since examination of the injection site over a period of one hour to six days afterward showed minimal inflammatory reaction and only a minimal amount of edema, which resolved in three days. There was noted significant initial tumor cell death that was compensated by new tumor cell formation taking six days for the number of tumor cells to return to the number seen one hour after injection. This study also emphasized the importance of counting the cells in the infusate before and after injection to make certain that the desired number of cells had been delivered. As noted in two of our 32 animals, it appeared that the cannula was occluded precluding the injection of tumor cells. The survival times of our animals is longer in this study compared with other reports [1, 6, 12, 14, 24], including this laboratory [1, 18], and most likely reflects the small volume and low number of cells that were injected.

Following tumor cell injection, there is a period of four to five weeks during which tumor volume very slowly increases. Evidence supports the concept that tumors are being nourished solely by perfusion during this interval. Studies have shown that injected brain tumor cells actively migrate toward and divide adjacent to pre-existing capillaries. These tumor cells erode into the capillaries which is then followed by angiogensis [2, 7-9]. Exponential growth of tumor is observed after approximately five weeks and is the result of the tumor developing its own blood supply. Studies are currently underway in this laboratory to further elucidate the mechanisms by which tumors stimulate angiogenesis as well as ways to prevent it from occurring as the inhibition of angiogensis would definitely influence the ability of tumors to grow.

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