Growth of Human Lung Tumor in the Brain of the Nude Rat as a Model to Evaluate Antitumor Agent Delivery across the Blood-Brain Barrier


ABSTRACT

We have developed a brain tumor model in the nude rat utilizing NCI-N417D human small cell carcinoma of the lung grown both intracerebrally and s.c. The median latency period from the time of intracerebral tumor inoculation to the onset of neurological symptoms is 13 days with an intracerebral tumor take rate of 91% (29 of 32). The median survival is 13 days, and all animals were dead by Day 26. The tumor is discrete, well circumscribed, with occasional leptomeningeal spread and with minimal evidence of surrounding cerebral edema. Intracerebrally, this tumor is usually impermeable to Evans’s blue:albumin (M, 68,500) but not fluorescein (M, 376). Although variable, the intracerebral tumor is less permeable to methotrexate than is the same tumor grown s.c. in the same animal (P < 0.005). The intraarterial and i.v. routes of methotrexate administration in the presence and absence of blood-brain barrier opening were evaluated. Drug delivery to the intracerebral tumor and ipsilateral brain was significantly (P < 0.025) greater when the methotrexate was given intraarterially and was significantly (P < 0.0025) increased after osmotic blood-brain barrier opening. After barrier opening, methotrexate concentration was enhanced 3- to 4-fold in tumor and 10- to 20-fold in brain around tumor. Thus, the nude rat provides a model to investigate the biology and therapeutic responsiveness of human small cell carcinoma of the lung grown intracerebrally where it develops a blood-tumor barrier similar to that seen in humans. This model further provides the unique opportunity to investigate the role of osmotic blood-brain barrier opening in the treatment of a tumor which is sensitive to cytoreductive chemotherapeutic agents that reflects the clinical effectiveness of these agents in the original patient. For instance, Giovannelli et al. (14) evaluated the efficacy of 9 chemotherapeutic agents on 3 types of human tumor xenografts implanted in the nude mouse (breast cancer, colon cancer, and melanoma); tumor regressions were consistent with the clinical experience in patients except for 2 false-positive and one false-negative values. Histological evaluation of human tumor xenografts has been reported to maintain morphological fidelity with the parent tumor (13). In addition, several reports (15–17) have shown that xenografts of human tumors can be consistently transplanted intracerebrally into the nude mouse, and Chambers et al. (18) have reported the intracranial growth of cells from small cell carcinoma of the lung in nude mice. One possible disadvantage of the nude mouse is that human tumors which have a high propensity to metastasize do not metastasize in nude animals.

INTRODUCTION

Although Greene (1, 2) reported heterotransplantation of human brain tumors in 1951, attempts to implant and grow spontaneous human tumors intracerebrally in laboratory animals have been inconsistent (3) and generally unsuccessful. Neither conditioning with immunosuppressive agents (4) nor utilization of the subarachnoid space of the animal as a growth chamber (5) provides a satisfactory model for the study of gliomas or other human tumors as xenografts. For instance, intracerebral growth of human glioma in immunosuppressed rats results in a highly permeable tumor (4) unlike the situation clinically (6, 7) or in a virally induced animal glioma model (8, 9).

The introduction of the athymic nude mouse (10) offered a new opportunity to study the growth and behavior of human tumor cells in vivo under controlled conditions (11). A variety of human tumors have been established and serially passed in the athymic mouse (12). The histology and responsiveness for most tumors in the nude mouse are similar to those in humans, and validation of this model for the study of a variety of biological features of human tumors has been achieved (13). Tumors so grown show a differential sensitivity to cytoreductive chemotherapeutic agents that reflects the clinical effectiveness of these agents in the original patient. For instance, Giovannelli et al. (14) evaluated the efficacy of 9 chemotherapeutic agents on 3 types of human tumor xenografts implanted in the nude mouse (breast cancer, colon cancer, and melanoma); tumor regressions were consistent with the clinical experience in patients except for 2 false-positive and one false-negative values. Histological evaluation of human tumor xenografts has been reported to maintain morphological fidelity with the parent tumor (13). In addition, several reports (15–17) have shown that xenografts of human tumors can be consistently transplanted intracerebrally into the nude mouse, and Chambers et al. (18) have reported the intracranial growth of cells from small cell carcinoma of the lung in nude mice. One possible disadvantage of the nude mouse is that human tumors which have a high propensity to metastasize do not metastasize in nude animals.

Unfortunately, the small size of the nude mouse makes it a very difficult model for the study of complex multimodality therapeutic approaches to intracerebral tumors. The ideal model would permit human xenografts to be examined at both intracerebral and systemic sites, and the intracerebral site should develop the biological characteristics of spontaneous primary or metastatic tumors, i.e., to demonstrate at least a partial BBB (8, 9, 19, 20). In addition, animal size should permit a variety of therapeutic manipulations, including i.a. (9, 21–23) infusions, opening of the BBB (6–8, 24, 25), and applicability for studies of “targeted” therapy, such as with monoclonal antibodies (26–29).

The nude rat provided the appropriate structural requirements, and human small cell (oat cell) xenografts provided the acceptable tumor for the development of a therapeutic model. The nude rat has immunological features like those of the nude mouse (30, 31). Thus, the congenitally athymic nude rat accepts allografts and xenografts, and their splenic lymphocytes respond

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The abbreviations used are: BBB, blood-brain barrier; MTX, methotrexate; CNS, central nervous system; i.a., intraarterial(y); i.c. intracarotid.
poorly to T-cell mitogens. Unlike the nude mouse, the nude rat is less susceptible to infections and is thereby easier to maintain. The nude rat does not provide as good a growth medium for tumor xenografts (31) as the mouse, perhaps as the result of a higher level of natural killer cells (32, 33). However, a variety of different human cancers (e.g., carcinoma of the breast, gastrointestinal tract, melanoma, and rhabdomyosarcoma) have been grown in the nude rat (34, 35).

The present studies focused on the small cell carcinoma (oat cell) of the human lung, since this carcinoma is a common tumor which frequently metastasizes to the CNS and is responsive to a variety of chemotherapeutic agents. In addition, a number of tumor-specific monoclonal antibodies have been developed to this neoplasm (36, 37). The human small cell tumors were grown in the nude rats, and histology, survival, and the delivery of a chemotherapeutic agent (MTX) were examined. These studies have shown the feasibility of the nude rat as a xenograft model to examine this common human tumor and to evaluate strategies for therapy by comparing the delivery of drugs to the tumor in the brain with the tumor at s.c. sites. In particular, this model allows an evaluation of the role of BBB modification in anti-tumor agent delivery.

MATERIALS AND METHODS

Nude Rat Colony. The animals utilized were the athymic nude rat (nu/nu) which were bred from an outbred pair (nu/+ ) furnished from Harlan Sprague Dawley (Indianapolis, IN). The stock and breeding animals were kept in laminar flow contaminant hoods. Heterozygote males were paired with heterozygote (nu/+ ) females which resulted in 50% homozygote nude (nu/nu) athymic animals. All animals received sterilized fat-enriched food and vitamin-supplemented water. Water and bedding were changed twice weekly under sterile conditions. Animals were examined daily.

Human Tumor Cell Line. The human tumor cell line used was the NCI-N417D (38) which originated in the laboratory of Dr. John D. Minna. Cells were grown in 20 ml of RPMI Medium 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin (10,000 units/ml), streptomycin (10,000 μg/ml), and gentamycin (50 μg/ml). Complete medium was filtered (0.2-μm Millipore) prior to use. All cells were maintained in 5% CO₂ at 37°C and passaged in culture twice weekly. The NCI-N417D human tumor cells are free-floating cell suspensions that are easily harvested by centrifugation at 250 × g (1200 rpm) for 3 to 5 min. Cell suspensions for inoculation were made by resuspending the cell pellet in medium to achieve a final concentration with a packed cell volume of 20 ± 1% as measured in a microhematocrit tube. Cell viability was evaluated by trypan blue exclusion; viability was greater than 85%.

Implantation of Human Tumor Cells into the Nude Rat. Implantation of NCI-N417D cells was achieved by the s.c. injection into the right flank of 750 to 1000 μl of tumor cell suspension with a packed cell volume of 20 ± 1% (approximately 20 to 27 × 10⁶ cells). Intracerebral implantation of tumor cells was performed after the rats were anesthetized by i.p. injection of sodium pentobarbital (50 mg/kg). The head was shaved, sterilized with iodine solution, and immobilized in a rat stereotaxic frame (David Kopf Instruments, Tujunga, CA). A 2-cm midline incision was made to expose the frontal bone on the right, where a 2-mm burrhole with a Stryker drill was placed just posterior to the coronal suture line and 4 mm lateral to the sagittal suture. A micromanipulator (Brinkman Instruments, Westbury, NY) equipped with a 27-gauge needle and a 100-μl syringe was used to inject 15 μl of tumor cell suspension with a packed cell volume of 20 ± 1% over 2 to 3 min at a depth of 1.5 mm. This concentration of cells equals approximately 4 × 10⁶ tumor cells. The wound was closed in a single layer.

BBB Opening in the Nude Rat. BBB opening in the nude rat was performed and graded as previously described (8). Five min before BBB opening, Evans blue (2%, 2 ml/kg, i.v.) and/or fluorescein (10%, 0.12 to 0.25 ml, i.v.) was administered.

MTX Delivery to Human Tumor in Nude Rats. Animals symptomatic from intracerebral tumor were randomly divided into 4 groups: Group I, i.v. MTX after i.e. 0.9% NaCl solution (saline) (BBB intact); Group II, i.c. MTX after i.c. saline (BBB intact); Group III, i.v. MTX after i.c. mannitol (BBB opened); or Group IV, i.c. MTX after i.c. mannitol (BBB opened). In all groups, MTX (4 mg/kg) was administered immediately after i.e. saline or mannitol over 1 min. Thirty min after MTX administration, a serum sample was obtained, and the animals were sacrificed with an overdose of sodium pentobarbital. Tumor, brain around tumor (1 to 2 mm of brain immediately surrounding tumor), brain distant to tumor (coronal section of the occipital lobe in the perfused hemisphere as well as in the contralateral brain (coronal section of occipital lobe) s.c. tumor, and other organs and tissues were obtained for MTX determinations. MTX content in serum and tissue was determined by radioimmunoassay as previously described (22).

The effect of the intracerebral implantation technique on BBB integrity was examined in rats inoculated with lethally irradiated NCI-N417D cells (8000 to 9000 rads, using a 137Cs source). On Day 14 after intracerebral inoculation, Evans blue, fluorescein, and MTX were administered i.v. as described above.

Statistical Analysis. MTX levels are expressed in ng/g of tissue or ng/ml of serum as the mean ± SE. Comparisons of the log concentration of tissues in the irradiated inoculum control group (Table 1) and within the 4 treatment groups (Table 2) were made by the one-way analysis of variance followed by the Neuman-Keuls multiple comparisons when the overall difference was statistically significant. The 2-factor analysis of variance was utilized to evaluate the effect of route of MTX administration and/or osmotic BBB opening on delivery to brain tissues (Table 2, Groups I to IV) (39, 40).

RESULTS

Survival and Onset of Neurological Symptoms. For the study of survival characteristics, 4 × 10⁶ human small cell carcinoma of the lung cells (serially passaged in tissue culture) were injected intracerebrally into the right parietal lobe of 32 nude rats. The median survival was 13 days (range, 10 to 25 days), and all animals were dead by Day 25 (Chart 1A). Because of the large variation in time from death to autopsy, intracerebral tumor confirmation was often not available in these rats. Of 32 additional rats given injections of intracerebral NCI-N417D human tumor cells, the median latency period from intracerebral inoculation to the development of neurological symptoms was also 13 days (range, 9 to 26 days) (Chart 1B). Symptoms identifying the presence of tumor included cachexia, weight loss, and dehydration. Animals died within 24 h of evident symptoms. Of these rats, 91% (29 of 32) developed verified intracerebral tumors. In the remaining 3 animals, intracerebral tumor could not be confirmed by visual inspection, but histology was not performed.

Pathological Characteristics. When the animals become neurologically symptomatic or at expiration, the tumors exhibited a mass effect on the brain. The tumors were approximately 5 to 7 mm in diameter with occasional leptomeningeal spread. No animal had multiple tumors. The tumors were usually subcortical and often extended to the ependymal wall of the lateral ventricle. The intracerebral tumors were round and had neovascularity. The tumor margin was clearly defined, and little surrounding cerebral edema was present (Fig. 1A). Systemic spread (other...
animals had symptoms by Day 26. Ninety-one % (29 of 32) had confirmed cell carcinoma of the lung cells. The median latency period from intracerebral neurological symptoms after intracerebral inoculation with NCI-N417D human small and all animals were dead by Day 25. B, curve illustrating the development of period from intracerebral inoculation to death was 13 days (range, 10 to 25 days), and all animals had symptoms by Day 26. Ninety-one % (29 of 32) had confirmed intracerebral tumor; the remaining 3 could not be confirmed by visual inspection, and histology was not performed.

than the simultaneously inoculated s.c. flank tumor has not been observed. Microscopic examination of the tumor cells shows them to be slightly larger than the characteristic human lung small cell tumor as identified in clinical material; mitoses were very frequent (Fig. 1B).

In the initial studies, the same size of tumor inoculum was used intracerebrally and s.c. However, at the time the animals became symptomatic from their intracerebral tumor, no s.c. tumor was detected when the tumor inoculum was the same at both sites. When s.c. tumor inoculum was increased 50- to 65-fold, the diameter of the s.c. tumor was 2 to 3 cm at the point when the animals developed neurological symptoms from the intracerebral tumor. The s.c. tumor was 4 to 5 times larger than the intracerebral tumor. In addition, in almost half the animals, the s.c. tumors were partially cystic and contained serosanguineous fluid.

Tumor Staining with Fluorescein and Evans Blue:Albumin. In the preliminary studies, it was often difficult to identify discrete tumors at the time of death, even when Evans blue (which binds tightly to albumin, thereby identifying barrier integrity) had been given. Thus, there were several animals in which the intracerebral tumor was missed by visual inspection, but confirmed histologically. This led to the use of i.v. fluorescein (M, 376) when symptoms were evident, thus providing clearly demarcated, easily visible tumors identified by the yellow stain of fluorescein (Fig. 2A). The relative integrity of the BBB was evaluated by the concurrent administration of i.v. fluorescein (M, 376) and Evans blue (Evans blue:albumin, M, 68,500). As shown in Fig. 2A, the intracerebral tumor usually stained with the low-molecular-weight moiety fluorescein, but not with the high-molecular-weight Evans blue:albumin, whereas both markers generally penetrated s.c. tumor (Fig. 2B). By contrast, when these same molecular markers (fluorescein and Evans blue:albumin) were given i.v. and osmotic barrier opening of the tumor-bearing hemisphere was performed, both Evans blue and fluorescein penetrated the tumor as well as the surrounding brain. A green discoloration (due to the combination of yellow and blue) of these tissues was seen in the area of brain where barrier opening was performed (Fig. 2, C and D). When only Evans blue was given i.v. at the time of barrier opening, the tumor and the brain around the tumor were stained blue.

MTX Delivery to Intracerebral and s.c. Tumor. To determine the effect of the inoculation technique on BBB integrity, lethally irradiated tumor inoculum was implanted intracerebrally. There was no evident staining with either i.v.-administered Evans blue or fluorescein. Following i.v. MTX administration, MTX delivery to the area of inoculation resulted in levels ranging between 0.5 and 3% of serum levels, which was consistent with MTX levels in the contralateral hemisphere of all inoculated animals (Table 1). There was a statistical difference (P < 0.05) in MTX concentration between the inoculation site (94 ± 54) and brain distant to the inoculation (16 ± 3) site and contralateral brain (17 ± 3). Despite an equivalent dose of MTX, the serum drug levels in tumor-bearing animals were higher than in animals receiving irradiated tumor cells, which probably reflects cachexia and volume depletion of tumor-bearing animals.

Drug delivery studies were begun when the animals manifested symptoms from their intracerebral tumor. Rats with both intracerebral and s.c. tumor were given MTX either i.v. or i.a. after either i.e. saline (Groups I and II) or mannitol infusion (Groups III and IV). After i.v. MTX, the intracerebral tumor in Group I contained significantly (P < 0.005) less MTX than the s.c. tumor, suggesting limited entry of drug at the intracerebral site (Table 2). The level of MTX in intracerebral tumor, although variable, was approximately 8% of that in serum, whereas the level in the s.c. tumor was approximately 20% of the serum level at the time of sacrifice. The intracerebral tumor, however, contained significantly more MTX than the brain around tumor, brain distant to tumor, and contralateral brain (P < 0.005).

MTX concentration in the intracerebral inoculation site of lethally irradiated small cell carcinoma tumor cells

<table>
<thead>
<tr>
<th>MTX (ng/g)</th>
<th>Tumor site</th>
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<tbody>
<tr>
<td>Inoculation site</td>
<td>94 ± 54± 25</td>
</tr>
<tr>
<td>Brain around inoculation site</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Brain distant to inoculation site</td>
<td>17 ± 3</td>
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<tr>
<td>Brain contralateral to inoculation site</td>
<td>3033 ± 33</td>
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*MTX, 4 mg/kg, was administered i.v. on Day 14 after the intracerebral inoculation. Animals were sacrificed 30 min after MTX administration (n = 3).

Significantly (P < 0.05) greater than brain distant to the inoculation site and brain contralateral to the inoculation site.
When the MTX was given i.a. after i.c. saline infusion (Group II), there was a 2-fold increase of MTX delivery to the tumor and brain around tumor as compared to Group I. Similarly, when MTX was given i.a. after i.c. mannitol (Group IV), there was a 3-fold increase to tumor and brain around tumor compared to Group III. Thus, route of MTX administration significantly (P < 0.025) altered drug uptake in tumor and surrounding brain. Osmotic BBB opening, independent of route of administration, also significantly (P < 0.0025) increased drug delivery to tumor, brain around tumor, and brain distant to tumor. MTX administered i.v. after osmotic opening (Group III) resulted in 2- to 9-fold increases compared to Group I. When MTX was administered i.v. after osmotic opening (Group IV), there was increased delivery of MTX, ranging from 2.5-fold in tumor to 30-fold in brain distant to tumor as compared with Group II. The greatest MTX delivery to tumor and ipsilateral brain was obtained after i.a. administration in the presence of BBB opening. By this route of administration, the MTX content in the intracerebral tumor was double that in the tumor at the s.c. site. The MTX levels in the s.c. tumor and serum were not statistically different in any of the groups.

Thus, less MTX entered this small cell carcinoma of lung grown intracerebrally in contrast to the tumor grown in s.c. tissues. Osmotic BBB opening combined with i.a. drug administration achieved drug levels in intracerebral tumor and surrounding brain equal to or greater than that found in the systemic tissues or s.c. tumor.

**DISCUSSION**

The critical requirement of an adequate xenograft model for the evaluation of complex multimodality therapeutic approaches to malignant intracerebral tumors is easily evident. For instance, in previous studies with the avian sarcoma virus-induced glioma in the rat, we have demonstrated that, by combining i.a. drug administration with reversible osmotic BBB opening, the delivery of MTX to the tumor and the brain around the tumor was increased over that achieved by routine (i.e., i.v.) routes of drug administration (8). However, the avian sarcoma virus-induced glioma model is not sensitive to most chemotherapeutic agents, and it is not amenable to an investigation of "targeted" therapeutic studies, since monoclonal antibodies have not been developed against these tumors.

The present studies utilized the immunologically athymic (nude) rat for the study of drug delivery to an intracerebral tumor. The rat was selected because its size permitted cannulation of the carotid artery necessary for i.a. drug administration and an examination of the effect of osmotic BBB opening on drug delivery. Although Colston et al. (31) reported that the nude rat was less able than the nude mouse to sustain a human tumor xenograft, we have had little difficulty in growing either human carcinoma of the breast or small cell carcinoma of the lung in this model. The present studies focused on the characterization of the growth of human small cell carcinoma of lung in the nude rat both intracerebrally and s.c.

This human small cell carcinoma of lung tumor model has provided several very important observations. The characteristics of this tumor model make it very suitable for the evaluation of antitumor agent (chemotherapeutic drug or monoclonal antibody) delivery in the presence or absence of BBB opening. A most important finding demonstrated in this model is that tumor inoculated and grown in the brain almost totally excludes (via the BBB) the uptake of a protein marker (Evans blue:albumin), even though there is partial uptake of smaller-molecular-weight markers (i.e., MTX and fluorescein). Evidence that this is not a property of the tumor cells is the greater uptake of Evans...
malignant brain tumors, permeability to drug was variable. As reported by Gazdar et al. (38), the dose of tumor cells necessary to produce a s.c. tumor was much greater than the dose necessary to produce an intracerebral tumor. The reason for this difference is not apparent.

In spite of these observations, the significance of the BBB in drug delivery to CNS tumors is still a matter of some controversy. Hiesiger et al. (24) recently stated that the blood tumor barrier in C57 brain gliomas is already open and cannot be further opened osmotically, thus explaining why MTX entry into such tumor does not increase after i.c. hyperosmolar mannitol. These observations are in contrast to our tumor model, which has a different permeability between the intracerebral and the s.c. tumor. Analogously, initial clinical trials of i.v.-administered 99Tc-glucoscheptanate and, at a different time point, 131I-tumor-specific monoclonal antibody resulted in positive scans with the low-molecular-weight 99Tc-glucoscheptanate but negative serial scans with the monoclonal antibody over a 10-day period. Thus, this patient's glioma, as with our tumor model, displays more permeability to low-molecular-weight than to high-molecular-weight markers. In addition, BBB opening increases the concentration of MTX delivered to the intracerebral tumor in both this model and our virally induced glioma model (8, 9).

It must be emphasized that the current studies only examine the question of drug delivery as a method to characterize this tumor, not drug efficacy. However, this model provides an exciting opportunity to evaluate therapeutic efficacy of osmotic BBB opening as a factor in therapy of cancers in the CNS, since human small cell carcinoma of the lung is very sensitive to chemotherapy (41), and since these are tumors to which highly specific monoclonal antibodies have been produced (36, 37). Because a steep dose-response curve seems to characterize most tumors (42) and often the proliferating edge of these tumors has an intact BBB (43), the utilization of osmotic modification to maximize antitumor agent delivery, be it drug or monoclonal antibody, seems very appropriate. The relatively brief and consistent latency period from the time of tumor inoculation to the onset of neurological symptoms further enhances the value of this model.

REFERENCES

NUDE RAT BRAIN TUMOR


Fig. 2. A, coronal section of a nude rat brain 17 days after 4 x 10^5 NCI-N417D human small cell lung carcinoma cells were implanted in the right hemisphere. Prior to sacrifice, Evans blue (2%, 2 ml/kg, i.v.) and fluorescein (10%, 0.12 ml, i.v.) were administered, followed by i.e. saline. Note the tumor is stained with fluorescein (M, 376) but not with Evans blue:albumin (M, 68,500). B, s.c. tumor in a nude rat 17 days after 27 x 10^5 NCI-N417D human small cell lung carcinoma cells were implanted. Fluorescein and Evans blue were given as in A prior to i.c. saline. Note that the tumor is stained by both Evans blue:albumin and fluorescein. C and D, intact (C) and coronal (D) section of a nude rat brain 18 days after 4 x 10^5 NCI-N417D human small cell lung carcinoma cells were implanted in the right hemisphere. Prior to sacrifice, Evans blue (2%, 2 ml/kg, i.v.) and fluorescein (10%, 0.12 ml, i.v.) were administered, followed by mannitol infusion into the right internal carotid artery. The tumor and surrounding brain in the distribution of barrier opening are stained with Evans blue:albumin and fluorescein (green due to blue and yellow).
Fig. 1. A, brain tumor interface of nude rat brain 23 days after $4 \times 10^6$ NCI-N417D human small cell lung carcinoma cells were implanted in the right hemisphere. There are neovascularity, a sharp tumor edge, and little surrounding cerebral edema. H & E, x 100. B, high magnification of section of Fig. 1A. The tumor cells are larger than the typical human small cell lung tumor, and mitotic figures are frequent. H & E, x 450.
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