Development of a Large-Animal Human Brain Tumor Xenograft Model in Immunosuppressed Cats


Division of Neurosurgery, Department of Surgery [B. W. K., M. A. F.-J., J. D. M., B. K. W., K. C. P.J, and Division of Neuropathology, Department of Pathology [B. M.J, University of Alberta, Edmonton, Alberta T6G 2G3, Canada

ABSTRACT

A large-animal model was developed to facilitate the noninvasive investigation of the effect on the human glioma-derived D-54 MG (glioblastoma multiforme) continuous cell line of a variety of therapeutic regimens. Twenty random-bred male cats were inoculated intracerebrally with 1 x 10⁶ D-54 MG tumor cells after being initiated on one of three preparatory regimens of cyclosporin A p.o. Reproducible success of D-54 MG xenotransplantation (100%, 6 of 6 cats) was achieved only after pretreatment with 120 mg cyclosporin A p.o. (24-30 mg/kg) daily for ≥10 days prior to tumor implantation. High-performance liquid chromatography-derived whole blood cyclosporin A 12-h trough levels of ≥640 ng/ml were seen in successful implants. Lesions ranging from 2 to 20 mm in diameter were seen in cats sacrificed 27–44 days after implantation with no growth seen in control animals.

Histopathological examination revealed the tumors to be well-circumscribed anaplastic intracerebral tumors with some invasion into surrounding host parenchyma. Perivascular lymphocytic cuffing was observed, but intratumoral lymphocytic infiltration was minimal. Gadolinium-EDTA-enhanced nuclear magnetic resonance imaging provided accurate tumor localization in T₁-weighted images (T₁ 26 ms; T₂ 600 ms). Biochemical tests of kidney, liver, and hematological function were within normal limits, although 10% (2 of 20) of the animals developed gingival hyperplasia, and 5% (1 of 20) developed intussusception.

The reproducible growth of the D-54 MG human glioblastoma cell line in a large-animal model eliminates many of the limitations associated with the standard nude mouse/rat model, thereby providing a novel test bed for a variety of imaging modalities as well as for drug immunoconjugate localization and toxicity studies.

INTRODUCTION

Primary central nervous system neoplasms constitute approximately 10% of all cancer-related admissions to hospital and represent the cause for approximately 1% of all human mortality in North America (1). Eighty percent of these primary tumors involve the brain, and fully one-half of these tumors are gliomatous. The more malignant types (malignant astrocytoma and glioblastoma multiforme) are clinically characterized by a rapidly progressive debilitating course with a uniformly fatal outcome. Current therapy consists of surgical biopsy and/or extirpation, followed by postoperative irradiation and chemotherapy (2). Despite this combined therapeutic assault, median survival rates have only marginally increased (3).

In the experiments reported here, we have observed the reproducible i.c. growth of the D-54 MG human glioblastoma cell line and the TE671 rhabdomyosarcoma cell line using CyA p.o. In addition, we have grown both tumor types in s.c. locations allowing for studies comparing the effect of the peri-tumor environment on tumor growth characteristics.

MATERIALS AND METHODS

Karyotyping. Karyotyping was performed to substantiate the similarity of the genotypic characteristics of the D-54 MG cell line as compared to previously published reports (4, 5). Colcemid (0.15 ml) was added to a T-25 flask of D-54 MG cells (passage 66) and incubated for 3 h at 37°C. Two ml of trypsin (0.25%) in Hanks' balanced salt solution were then added. After 3 washes with 2 ml of Dulbecco's modified Eagle's medium with 10% fetal calf serum, the suspension was placed in a centrifuge tube. After centrifugation of 1000 rpm for 10 min, hypotonic solution was added to the supernatant which was then placed in a 37°C water bath for 17 min. The mixture was fixed with methanol/acetic acid and left overnight at 4°C. The solution was then spun and refixed three times. Four or five drops of fixed suspension were then dropped onto a cold, wet slide which was left overnight at 60°C. Slides were trypsinized for 15 s and then rinsed in 70 and 100% ethanol after which they were quickly dried. Giemsa stain (pH 6.8) was applied for 3 min, and thereafter the slides were rinsed twice, dried, and made ready for examination.

Pharmacokinetic Study. Three randomly bred cats were sedated with ketamine i.m. (Parke-Davis, Morris Plains, NJ) (20 mg/kg) and catheterized via the internal jugular vein. Cats were given 60 mg CyA p.o. presedation. Blood samples were then taken i.v. at 0, 1, 2, 3, 5, 7, 10, 12, 24, 36, and 48 h. An exponential curve-fitting analysis program was utilized to determine the half-life of CyA p.o. in cats.

Animals and CyA Treatment Protocol. Twenty randomly bred adult (≥1 year) male cats weighing 3.5–5 kg were quarantined for 21 days after receipt. All animals tested negative for feline leukemia virus and were given no immunization. Animals were fed ad libitum. Cyclosporin A (courtesy of Sandoz Canada, Inc.) was administered as Sandimmune oral solution (cyclosporin A, 100 mg/ml in Labrafil/olive oil). This solution was then transferred to commercially available empty cod liver oil capsules (2 sizes, ~0.45 and ~0.9 ml) into which 0.4 ml (40 mg) and 0.8 ml (80 mg) of CyA solution were placed, respectively. After quarantine, cats were placed into one of four treatment groups: Group 1, 80 mg CyA/day p.o. in 2 divided doses, starting 24 h preimplant (6 cats); Group 2, 120 mg CyA/day p.o. in 2 divided doses (80 mg every morning plus 40 mg every evening), starting 24 h preimplant (5 cats); Group 3, 120 mg CyA/day p.o. in 2 divided doses, starting ≥10 days preimplant (6 cats); Group 4, 120 mg placebo solution/day (olive oil carrier solution for oral preparation; courtesy of Sandoz Canada, Inc.) in 2 divided doses, starting 10 days preimplant (3 cats). An additional...
8 cats were split into the following three groups: Group 5, i.c. implanted TE671 human rhabdomyosarcoma (80 mg CyA p.o. in 2 divided doses, 24 h preimplant), 2 cats; Group 6, TE671 implanted s.c. (80 mg CyA p.o. in 2 divided doses, 24 h preimplant), 2 cats; Group 7, D-54 MG implanted s.c. (same dose as s.c. TE671), 4 cats. All animals receiving CyA underwent weekly blood tests to monitor liver function (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total protein, bilirubin, calcium, and magnesium), kidney function (blood urea nitrogen and creatinine), and hematological function (hemoglobin, hematocrit, and complete blood count with differential). In addition, the 6 cats receiving 120 mg CyA/day p.o. starting 10 days preimplant (Group 3) had CyA trough levels drawn at days 10 and 17. Trough levels were determined on heparinized whole blood samples using a Clia reverse-phase high-performance liquid chromatography column, with cyclosporin D as an internal standard (5).

Tumor Implantation. D-54 MG human glioblastoma-derived continuous cell line and TE671 human rhabdomyosarcoma (both cell lines were supplied by courtesy of Dr. D. Bigner, Duke University) were used in all implants. Both were cultured in RPMI 1640 with 10% fetal calf serum and antibiotics (penicillin/streptomycin). Passages 66 through 70 of D-54 MG were used in i.c. and s.c. implants. Passages 218 through 220 of TE671 were also used for implantation. After an overnight fast, animals were anesthetized with Saffan (Alphaxalone- Alphadolone; courtesy of Glaxo, Ltd.) using an induction dose of 0.75 ml/kg (9 mg Alphaxalone and 3 mg Alphadolone/ml solution injected over a 1-min period). The animals were intubated, and a 20-gauge catheter was inserted into the cephalic vein. The animals were subsequently maintained on doses of 0.5 ml titrated to respiratory rate and continuously monitored. The animals were subsequently maintained on doses of 0.5 ml titrated to respiratory rate and corneal reflex elicitation. Their heads were shaved, positioned in a stereotactic head holder, and cleansed with providone-iodine solution. A 2.5-cm midline scalp incision was made. Two 5-mm-diameter burr holes were made, one on each side of the midline, 5 mm posterior to the coronal suture. D-54 MG cells (1 x 10^7), previously trypsinized and pelleted by gentle centrifugation at 1000 rpm for 5 min and then mixed in equal volumes with 1% methyl cellulose in RPMI 1640, were inoculated in a 100-μl volume, 5 mm deep to dura into each burr hole, through a Hamilton syringe fitted with a 22-gauge needle, over a 3-min period. Cells were implanted bilaterally to increase the tumor take rate per animal. Delay time between inoculations was 5 min. Cells were allowed to spill out subpially over the cortical surface. Both burr holes were sealed with bone wax, and the incision was closed with interrupted sutures. Cats were then monitored twice daily for tumor- or CyA-related signs and painlessly overdosed with Pentothal i.v. after NMR imaging, when signs of neurological deterioration appeared. Animals not spontaneously presenting with signs were overdosed 82 days postimplant.

Histopathological Examination. Cat brains were removed within 2 h after sacrifice, and gross morphological characteristics were noted. Brains were fixed in 10% formaldehyde for ≥24 h and then cut in coronal section. Sections were paraffin embedded and cut into 8-μm serial slices which were then stained with hematoxylin and eosin. Sections were examined for tumor presence and dimensions, cellular morphology, and extent of lymphocytic infiltration. Statistical analysis was performed using the chi-square test with the Yates continuity correction for small sample sizes; P < 0.05 was considered statistically significant.

NMR Imaging. A single-slice, multiecho experiment was performed on a cat at least 21 days after implantation using a 2.35-T Bruker 40-cm-bore magnet. Eight echoes in the spin-echo experiment were recorded (T2, 26.5 ms; T1, 3 s) after centering on the position at which the greatest tumor diameter was observed. Furthermore, 1 ml Gd-DTPA (courtesy of Berlex Canada, Inc., Lachine, Quebec, Canada) was administered i.v. over 2 min to several additional cats (4.4–5.0 kg) and implanted after the main studies were completed, in an attempt to increase delineation between tumor and surround (T2, 26.5 ms; T1, 0.5 s for this small series).

RESULTS

Karyotyping. A determination of chromosome number was performed on 50 D-54 MG tumor cells in culture. Seventy percent of these cells had counts of 70–73 chromosomes; however, some cells had fewer than 68 chromosomes and others had a greater number, up to a maximum of 76 chromosomes (Fig. 1). Five of these cells, taken from the midrange of chromosome number, were karyotyped, and a representative set of marker chromosomes is shown in Fig. 2. This set contains 15 of 18 marker chromosomes previously described for this tumor cell line, and both the karyotype as well as the distribution of chromosomal markers compare favorably to previously published reports (5, 6).

Pharmacokinetic Study. This study was performed to ascertain the appropriate dosing schedule for cyclosporin A p.o. The time to peak concentration for a 60-mg dose p.o. in all 3 cats was 3 h. A representative study is shown (Fig. 3). The average half-life of CyA in blood was determined to be 13.6 ± 2.5 h (SD).

Tumor Implantation. Thirty-three percent (2 of 6) of cats implanted i.c. with D-54 MG tumors, on a preparatory regimen of 80 mg CyA/day p.o. commencing 24 h preimplant (Group 1), grew macroscopic tumors. In Group 2, with an increased dose (120 mg/day) for an equivalent length of time (24 h), the take rate was 60% (3 of 5 animals). Group 3 animals, exposed to a high-dose regimen (120 mg/day) for an increased length of time (≥10 days), grew tumors in 100% of the cases (6 of 6 animals), although the actual tumor take rate was only 75% (9 of 12 inoculations; P = 0.013). All cats in this last group achieved growth of right-sided implants. However, cells implanted in the left hemisphere invariably grew into smaller tumors and in 50% of cases (3 of 6) did not establish tumor nidi. A case-by-case synopsis of this last series can be found in Table 1. None of the 3 placebo-fed cats (Group 4) developed tumors, on a macroscopic or microscopic level (0 of 6 inoculations). The cat autopsied earliest (due to the appearance of signs of increased intracranial pressure), on coronal sectioning, revealed a cystic tumor with a necrotic center (B-1; 15 days); no other tumors had such a necrotic focus. The 2 cats implanted i.c. with TE671 rhabdomyosarcoma (Group 5) grew tumors; both died of mass effect 13 and 17 days after implantation. A low-power hematoxylin and eosin section of brain revealed the necrotic center of the tumor, which, on gross examination, was filled with a viscous red-tinged fluid.

All animals received CyA dosing from initiation of treatment until sacrifice or death. Two cats implanted into the right flank with TE671 cells (Group 6) grew tumors, which, after 28 days, averaged 1.5 to 2.0 cm in diameter. On sectioning, these tumors were revealed to have invaded underlying muscle but not overlying skin. Of the 4 cats implanted s.c. with D-54 MG cells (Group 7), only one developed a macroscopic tumor (2 cm in diameter) at 17 days. The dose of CyA was 2431
LARGE-ANIMAL HUMAN GLIOMA XENOGRAFT MODEL

D-54 MG MARKER CHROMOSOMES

Fig. 2. Marker chromosomes found in a representative D-54 MG cell (grown in tissue culture). Roman numerals, chromosomes previously assigned; DM, double minute; frag, unassigned chromosomal fragment.

uncharacterized chromosomes

DM frag

Table 1 Analysis of the 6 cats receiving 120 mg/day CyA, starting ≥10 days preimplant (Group 3)

| Cat   | Time until sacrifice (days) | CyA dose (mg/day) | Body wt (kg) | Trough [CyA] (mg/liter) on days 10 and 17 | Tumor dimensions (mm)
|-------|-----------------------------|-------------------|--------------|------------------------------------------|------------------------
| A-143 | 38                          | 120               | 4.6          | 2.24, 3.10                               | R: 2 x 2 x 2 L: No growth |
| A-162 | 27                          | 120               | 4.3          | 2.95, 2.42                               | R: 5 x 3 x 2 L: No growth |
| B-1   | 15                          | 120               | 4.8          | 0.75, 0.64                               | R: 12 x 8 x 8 L: No growth |
| B-14  | 44                          | 120               | 3.8          | 0.93, 2.91                               | R: 20 x 10 x 5 (cystic) L: 20 x 10 x 5 |
| B-2   | 38                          | 120               | 4.1          | 2.93, 4.60                               | R: 20 x 10 x 5 L: 20 x 10 x 1 |
| B-30  | 41                          | 120               | 4.1          | 0.94, 1.45                               | R: 20 x 10 x 12 L: 3 x 5 x 2 |

* Anteroposterior x lateral x depth.

diameter), at 28 days postimplantation. The other 3 implants initially developed macroscopic tumors which had a peak diameter of approximately 3 cm at 2 weeks postimplant but underwent progressive diminution in size until, at 4 weeks, there was neither macroscopic nor microscopic evidence of tumor tissue. All biochemical parameters tested were within normal limits, although 2 of 20 (10%) and 1 of 20 (5%) of the animals developed gingival hyperplasia and intussusception, respectively. The minimum CyA 12-h trough level in Group 3 was 640 ng/ml.

NMR Imaging. There was considerable difficulty resolving tumor tissue on the first echo of a multiecho NMR image (Fig. 4), but by the second and especially the third echo, even with the general disappearance of signal, one could clearly delineate tumor/edema from normal brain, although differentiation of tumor from surrounding edema still remained a matter for speculation. Gd-DTPA markedly increased the contrast (cf. pre- and postinjection; Fig. 5) in the area in which blood-brain barrier breakdown occurred, which, according to previous studies (7, 8), represents the main body of the tumor.

Histopathological Examination. On gross examination the tumors resulting from the D-54 MG implants were pale yellow, firm, and distinguishable in texture from surrounding parenchyma. Histologically, the neoplasms extended from the cerebrum into the subarachnoid and subdural spaces. In some cases there was invasion into the overlying subcutis as well. The subdural extension was in most cases greater in the anterior-posterior plane from the implant site than in the lateral direction. There was extensive invasion into surrounding brain. The histomorphology of the tumors was characterized by solid
sheets of polygonal cells interspersed with occasional giant cells. Mitoses were frequent throughout (Figs. 6 and 7). Some perivascular lymphocytic cuffing was present and was associated with minimal penetration into the tumor tissue in some cases but was completely absent in most cases.

It was interesting to note that in an animal that expired 3 days after implantation (prior to the commencement of the study) no tumor cells were present within the injection track, but there was an extensive infiltration of lymphocytes and fibroblasts at the site of injury. There were, however, numerous, viable D-54 MG cells in the subarachnoid region surrounding this site, many with mitotic figures. Differences were also seen
Fig. 5. Four coronal NMR images of cat brain (B-116) with a single right-side D-54 MG implant taken before (A), and 2, 5, and 10 min after (B-D) Gd-DTPA contrast enhancement. Imaging parameters: $T_1$, 26.5 ms; $T_2$, 0.6 s (first echo shown in each case).

Fig. 6. Photomicrograph of D-54 MG glioblastoma multiforme implanted into cat brain showing interface between tumor and brain. Tumor can be seen on the left of the figure, invading normal brain. H & E stain ($\times$ 115).
between D-54 MG tumors grown in s.c. and i.c. sites. Whereas sheets of closely cohesive polygonal cells were observed in tumors implanted i.c., the tumors implanted s.c. had a more sarcomatous appearance with whorls and haphazard arrangements of fusiform cells as well as striated organization of cell sheets.

One cat was also implanted (prestudy) with both D-54 MG as well as TE671 cells, both of which grew into macroscopic tumors. On microscopic examination these neoplasms showed distinct differences, with the TE671 lesion being much more densely populated with anaplastic, pleomorphic cells, as well as containing multifocal areas of necrosis (Fig. 8).

**DISCUSSION**

Numerous attempts have been made to establish a replicable model of a human tumor in an immunocompetent large animal, going as far back as 1951 (11). Various methods have been utilized to overcome the response of the host immune system to the insertion of a nidus of foreign tissue, including the use of: (a) immune-protected areas for implantation such as cheek pouch [hamster (12)] and the anterior chamber of the eye [rabbit (13, 14)], among others (15–17); (b) exogenous immunosuppression, including total body irradiation (10), antithymocyte serum (10), cyclophosphamide (18), and steroids (10, 19); (c) implantation of a fast-growing tumor into a partially im-
munoprotected area, such as human choriocarcinoma in brain [monkey (20)]. However, the great majority of these attempts were not successful, and the remainder proved to have limitations with respect to the size of tumor produced or the incongruity of the host implant tissue type when compared with the site of origin of the implanted human tumor.

The advent of cyclosporin A (21) has radically altered both the survival of the organ transplant recipient and the scope of studies on the rejection of grafts of various tissue types. Cyclosporin A is a fungal metabolite which binds to peptidyl-prolyl cis-trans-isomerase, which in turn inhibits isomerase-dependent refolding of proteins (22). This may be one of the mechanisms that is necessary for the activation of transcriptional activators including those involved in the activation of lymphokine genes such as interleukin 2 and γ-interferon. Inhibition of the activation of these genes would therefore block production of these lymphokines, which are necessary for T-cell activation. The use of cyclosporin A in xenografting human neural tissue into the mammalian central nervous system began with Stromberg et al. (23) who grafted fetal human (6.5–8 weeks) neural tissue into rat brain, achieving 100% survival of grafted tissue at 140 days. Adams et al. (4) grafted D-54 MG glioblastoma into rat brains with a 70–100% tumor-related mortality in 20–44 days. Our results have shown the presence of viable D-54 MG tumors 2–20 mm in diameter in cat brain, 27–44 days after implantation in 100% of cases. In addition, these tumors, as well as TE671 human rhabdomyosarcoma, can be grown in both s.c. as well as i.c. locations.

The D-54 MG cell line was utilized because of previous work showing it to be the most tumorigenic glioma cell line of 15 cell lines tested (24). Male cats were used because of the appearance of a consistent gender discrepancy in weight loss when CyA was used in xenografted rats (4). Administration of CyA p.o. was chosen because of its ease of administration and the avoidance of problems associated with the i.v. vehicle Crepehomor EL, i.e., vasoconstriction and histamine release [cf. Albrechtsen et al. (25)]. One possible explanation of the uneven outgrowth distribution of bilaterally implanted tumor cells may be methodological, i.e., the time delay (approximately 5 min) between cells implanted first into the right hemisphere, followed by the left-side implant. Pelletting, as well as the addition of Methocel, may decrease the access to the nutrients present in the surrounding medium, perhaps contributing to in vitro cell death preimplantation.

The observation that tumor cells remain viable and initially grew only in the subarachnoid space with subsequent invasion into the cerebral hemispheres4 has led to the speculation that there may be trophic factors conducive to tumor growth present in the cerebrospinal fluid. From these studies, we suggest that there may be three major prerequisites for successful growth of human tumors in the mammalian central nervous system [cf. Bjorklund et al. (26)]: (a) adequate number of viable tumorigenic cells; (b) adequate level of immunosuppression present for a sufficiently long time prior to transplantation; (c) immediate access to a nutrient supply (e.g., cerebrospinal fluid), pending the establishment of a permanent blood supply.

The question of whether or not xenografted tumors are capable of withstanding host cell-mediated immune responses after withdrawal of exogenous immunosuppression also awaits studies in progress.

The large-animal human glioma model presents several potential advantages over the currently used athymic mouse/rat model. These include: (a) larger cranial vault volume allowing for increased initial tumor cell implant volume and increased size of resultant tumor; (b) high host tolerance of cyclosporin A p.o. and a relative paucity of significant side effects; (c) facilitated visualization of (i) in vivo serial NMR images monitoring tumor growth and (ii) in vivo radioimmunocisthographic images utilizing tumor-specific monoclonal antibodies.

To summarize, the use of a large-animal model of human glioma confers the ability to test an augmented range of administration routes (e.g., intrathecal/intratumoral versus parental) and in vivo testing modalities, both invasive and noninvasive, that can be brought to bear in a study of brain tumor therapy. This may facilitate the development and ease the subsequent evaluation of innovative and efficacious approaches to this as yet incurable condition.

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4 Unpublished observations.


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