Published in final edited form as:

Neurosurgery. 2007 January ; 60(1): 167–178.

Adenoviral-mediated Gene Transfer into the Canine Brain In Vivo

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Abstract

OBJECTIVE: Glioblastoma multiforme (GBM) is a devastating brain tumor for which there is no cure. Adenoviral-mediated transfer of conditional cytotoxic (herpes simplex virus [HSV] 1-derived thymidine kinase [TK]) and immunostimulatory (Fms-like tyrosine kinase 3 ligand [Flt3L])

transgenes elicited immune-mediated long-term survival in a syngeneic intracranial GBM model in rodents. However, the lack of a large GBM animal model makes it difficult to predict the outcome of therapies in humans. Dogs develop spontaneous GBM that closely resemble the human disease; therefore, they constitute an excellent large animal model. We assayed the transduction efficiency of adenoviral vectors (Ads) encoding β -galactosidase (β Gal), TK, and Flt3L in J3T dog GBM cells in vitro and in the dog brain in vivo.

METHODS: J3T cells were infected with Ads (30 plaque-forming units/cell; 72 h) encoding β Gal (Ad- β Gal), TK (Ad-TK), or Flt3L (Ad-Flt3L). We determined transgene expression by immunocytochemistry, β Gal activity, Flt3L enzyme-linked immunosorbent assay, and TK-induced cell death. Ads were also injected intracranially into the parietal cortex of healthy dogs. We determined cell-type specific transgene expression and immune cell infiltration.

RESULTS: Adenoviral-mediated gene transfer of HSV1-TK, Flt3L, and β Gal was detected in dog glioma cells in vitro (45% transduction efficiency) and in the dog brain in vivo (10-mm² area transduced surrounding each injection site). T cells and macrophages/activated microglia infiltrated the injection sites. Importantly, no adverse clinical or neuropathological side effects were observed.

CONCLUSION: We demonstrate effective adenoviral-mediated gene transfer into the brain of dogs in vivo and support the use of these vectors to develop an efficacy trial for canine GBM as a prelude to human trials.

Keywords

Adenovirus; Dog; Flt3L; Ganciclovir; Gene therapy; Glioblastoma; HSV1-TK

Glioblastoma multiforme (GBM), the most common type of primary brain tumor in adults, is an aggressive and locally invasive tumor. Survival of patients affected by GBM has remained virtually unchanged during the past decades (6–12 mo after diagnosis) despite advances in surgery, radiation, and chemotherapy (8,12,16,24,27,37). Considering the poor prognosis of patients with high-grade glioma, novel therapeutic approaches are needed. Gene therapies are currently being developed and implemented in rodent preclinical models and in clinical trials (2,13,21–23,26,38). However, the lack of a large animal model of GBM makes it difficult to predict the outcome of preclinical therapies when translated directly from small rodent brains to those of human patients.

Promising results in preclinical rodent experiments have led to human clinical trials, some of which showed therapeutic efficacy (13,26,40,50). Nevertheless, the lack of a preclinical model that closely mimics the human condition and neuroanatomy, in addition to host immune responses to the gene therapy vectors, has hampered the clinical implementation of novel therapies. The small size of the rodent brain does not allow accurate optimization of volumes and doses that will be suitable for human GBM patients. It is likely that the different sizes of rat (2 g) and human (1300–1500 g) brains may contribute to discrepancies in preclinical efficacy studies and results obtained in human trials.

Our laboratory has shown efficacy in eradicating GBM using an immunostimulatory therapy in large, intracranial syngenic GBM model in Lewis rats in which the tumor occupies most of the volume of the striatum in one hemisphere by using adenoviral-mediated gene transfer of herpes simplex virus (HSV1) 1-derived thymidine kinase (TK) and Fms-like tyrosine kinase 3 ligand (Flt3L) (1,2). Because one of our aims is to translate this approach to human patients, we implemented it in dogs with spontaneous GBM, which may better model human disease parameters.

Dogs have large brains (72 g in beagles) compared with rodents. This makes them an attractive model to test gene delivery efficiency, evaluate vector and transgene biodistribution, and

optimize treatment protocols before clinical implementation in humans. Dogs have already proven to be an excellent model of human Duchenne muscular dystrophy (9,11). Furthermore, brachycephalic breeds, such as Boston terriers and boxers (20,25,29), are predisposed to develop spontaneous glioblastomas that closely resemble the human disease (44,45). Clinical signs, imaging, histopathology, treatment, and prognosis of human and canine GBMs are similar. In addition, there is a high correlation of neuroimaging features observed with magnetic resonance imaging scans in canine and human GBM, which is used as a diagnostic tool for canine GBM. Peritumoral edema is a consistent finding in both human and canine GBM (30, 35).

Canine GBMs are histopathologically indistinguishable from human GBMs (30). Both are highly infiltrative, contain areas of intratumoral necrosis, pseudopalisading, hemorrhage, cellular pleomorphism, nuclear atypia, abnormal mitosis, and endothelial proliferation (34). Thus, dogs bearing spontaneous GBM constitute an excellent large animal model to test efficacy and toxicity of novel glioma therapies. In this article, we demonstrate that adenoviral vectors (Ads) Type 5 can transduce canine glioma cells in vitro and the dog brain in vivo. We tested the transduction efficacy in dog J3T dog glioma cells infected with adenoviral-encoding therapeutic transgenes, such as HSV1-TK and Flt3L, or the reporter gene, β -galactosidase (β Gal).

Our immunotherapeutic approach combines the cytotoxic properties of HSV1-TK, which kills glioma cells in the presence of ganciclovir (GCV) (5,32) and causes the concomitant release of tumor antigens, with the immunostimulatory effects of Flt3L (15), which attracts antigen-presenting cells to the brain and the tumor mass (1,2,15). This combined approach is efficient in eliminating large, intracranial GBM in the syngenic Lewis rat model, in which all other therapies fail (2). We administered Ads in vivo in the brain of dogs and determined transgene expression in neurons and astrocytes, as well as limited infiltration of immune cells to the injection site. We demonstrate effective adenoviral-mediated gene transfer of HSV1-TK, Flt3L, and β Gal in dog glioma cells in vitro and into the normal dog brain in vivo. Importantly, no adverse clinical or neuropathological side effects were observed. This report constitutes an original description of adenoviral-mediated transgene expression into the brain of dogs in vivo and supports the use of these vectors to develop an efficacy trial of gene therapy for canine GBM. The results generated will be used as a prelude to implementing this novel therapeutic approach to treat human GBM patients.

MATERIALS AND METHODS

First-Generation Ads

The first-generation replication-defective recombinant Type 5 Ad expressed β Gal (Ad- β Gal), HSV1-TK (Ad-TK), or soluble human Flt3L (Ad-Flt3L) under the transcriptional control of the human cytomegalovirus intermediate early promoter (hCMV) embedded within the E1 region (42). The construction of these vectors has been described in detail previously (1,2, 17,48). The vectors were scaled up in our laboratory by infecting the human embryonic kidney HEK 293 cell line with a multiplicity of infection of 3 plaque-forming units (pfu)/cell of vector seed stock. The cells were harvested 48 hours later, lysed with 5% deoxycholate and deoxyribonuclease I, and Ads were purified by ultracentrifugation over two cesium chloride step gradients (36,42). Vectors were titrated in triplicate by end-point dilution, cytopathic effect assay (42). The titers were as follows: Ad-hCMV- β Gal (RAd35) (Ad- β Gal in this study) (17, 46,48,49) was 1.64×10^{11} pfu/ml; Ad-hCMV-HSV1-TK (Ad-TK in this study) (1,2) was 3.28×10^{11} pfu/ml; Ad-hCMV-hsFlt3L (Ad-Flt3L in this study) (1,2,15) was 2.05×10^{10} pfu/ml. The vector preparations were screened for the presence of replication-competent adenovirus (18,42) and for lipopolysaccharide contamination (Cambrex, East Rutherford, NJ) (14,42). Virus preparations used were free from replication-competent adenovirus and

lipopolysaccharide contamination. The expression of the transgenes encoded within the first generation and high capacity Ads was verified by infecting COS-7 cells with a multiplicity of infection of 100 viral particles/cell and performing immunocytochemistry with antibodies for β Gal, TK, and Flt3L as described below. Circulating antibodies to Ad were determined as described previously (53).

J3T Canine Glioblastoma Cells

The dog GBM cell line J3T (3,39) was obtained from Dr. Michael Berens (Translation Genomics Research Institute, Phoenix, AZ). Cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mmol/L L-glutamine, and 2 mmol/L non-essential amino acids. Cells were infected with a dose of 30 pfu/cell of Ad-TK, Ad-Flt3L or, as a control, Ad- β Gal under the control of the hCMV promoter. After 72 hours, cells were processed for immunocytochemistry, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) viability assay, or β Gal enzymatic activity assay, and the supernatants were collected for Flt3L enzyme-linked immunosorbent assay (ELISA).

Dogs

Healthy adult beagles were purchased by University of Minnesota Research Animal Resources from a licensed vendor and kept in a pathogen-free and well-maintained canine housing facility at the Veterinary Medical Center at the University of Minnesota. These experiments were conducted according to an approved Institutional Animal Care and Use Committee protocol and every effort was made to minimize pain and discomfort. The housing area was maintained at 12 hours light and 12 hours dark at $22 \pm 2^\circ\text{C}$ throughout the study. Every dog was fed ad libitum 220 g of standard maintenance diet daily. Blood from these dogs was obtained the day of surgery, 24 hours after surgery, and the day of sacrifice. Rectal body temperature was monitored daily for the duration of the study.

Anesthesia and Surgery/Gene Delivery

Before the surgery, the dogs were sedated with an intramuscular administration of 0.01 mg/kg acepromazine. An intravenous catheter was placed in a cephalic vein and anesthesia was induced with an intravenous administration of 5 mg/kg sodium thiopental. A cuffed endotracheal tube was placed, and a light anesthesia was maintained using 2% (end-tidal) isoflurane in 100% oxygen at a flow rate of 2 L/min. Lactated Ringer's solution (10 ml/kg/h) was administered intravenously during anesthesia. For postoperative analgesia, 2.2 mg/kg carprofen, a nonsteroidal anti-inflammatory drug, was orally administered every 12 hours, and 0.01 mg/kg buprenorphine was administered intravenously and subcutaneously every 4 to 6 hours, as needed.

A skin incision was made along the dorsal midline of the skull to expose the sagittal crest. The fascia of the superficial and deeper temporalis muscles were incised and the temporalis muscles elevated and reflected laterally to expose the skull from the nasion to the inion. Five holes were made in the skull using a 1.5-mm drill bit. The holes were centered between the nasion and inion, approximately 1.5 cm lateral to the sagittal crest, and spaced 1 cm apart. Ligatures of 3-0 polyglycolic acid suture (Vicryl; Ethicon, Inc., Somerville, NJ) and hemostatic forceps were used for hemostasis. An 18-gauge hypodermic needle was used to make a stab incision in the dura mater and the tip of the infusion catheter was advanced 2 cm into the cerebral cortex, directed in a paramedian plane.

Adenovirus was diluted with sterile saline to 8×10^8 pfu in a final volume of 50 μl . Five bilateral injections were administered; spaced 1 cm apart at a depth of 2 cm (from cortical surface) in 5 μl /site (8×10^7 pfu/injection site) during 5 minutes. Bone wax was packed around the catheter

in the drill hole to stabilize the catheter during infusion of the vectors. The temporalis fascia was apposed with 3–0 polydioxanone in a simple continuous pattern. The superficial musculature and subcutaneous tissues were closed with 3–0 polyglycolic acid in a simple continuous pattern and the skin edges reapposed with 3–0 nylon (Ethilon; Ethicon, Inc.) in interrupted cruciate sutures.

Monitoring and Euthanasia of Dogs

Body temperature was taken with a rectal thermometer. Blood and cerebrospinal fluid (CSF) from the cisternae magna were collected immediately before surgery and 3 days afterwards. Complete blood count, serum chemistry, urinalysis, and CSF analysis were performed by clinical pathology in the Veterinary Medical Center. Seven days after surgery, dogs were humanely euthanized by intravenous administration of 200 mg/kg sodium pentobarbital. After euthanasia, all dogs were perfused with freshly prepared Tyrode's solution (132 mmol/L NaCl, 1.8 mmol/L CaCl₂, 0.32 mmol/L NaH₂PO₄, 5.56 mmol/L glucose, 11.6 mmol/L NaHCO₃, and 2.68 mmol/L KCL) with 500 U of heparin with O₂ aeration by cardiac perfusion followed by perfusion with 4% paraformaldehyde. Cerebral hemispheres were removed and fixed in 4% paraformaldehyde in phosphate-buffered saline for 5 days.

βGal Activity

J3T dog glioma cells (5×10^4 /well) were infected with 30 pfu/cell of Ad-βGal. After 72 hours, protein extracts were prepared by three freeze-thaw cycles in phosphate-buffered saline containing a cocktail of protease inhibitors (Endogen). βGal activity was measured by conversion of o-nitrophenyl-β-D-galactopyranoside in 10 mmol/L MgCl₂ and 0.45 mol/L 2-mercaptoethanol, which was detected at 420-nm absorbance. βGal activity was expressed as enzymatic activity per minute (o-nitrophenol mg/ml/(time [min] × protein [mg/ml])).

Flt3L ELISA

J3T dog glioma cells (5×10^4 /well) were infected with 30 pfu/cell of Ad-Flt3L. After 72 hours, the supernatant was collected, cellular debris was removed by centrifugation, and the supernatant was stored at −70°C. Flt3L concentration was determined in 50 μl of J3T cell culture supernatant and in dog serum, before and 7 days after adenoviral administration, using a commercial ELISA kit (21–377–296; R&D Systems, Minneapolis, MN) and following manufacturer instructions (15).

HSV1-TK Cytotoxicity

J3T dog glioblastoma cells (5×10^4 /well) were infected with 30 pfu/cell of Ad-TK. After 72 hours, the cells were incubated for an additional 72 hours in the presence of 10 μmol/L of the prodrug, GCV. Cell viability was determined by the MTS assay (Promega, Madison, WI). Twenty microliters of reaction solution containing MTS (final concentration, 333 μg/ml) and an electron-coupling reagent (phenazine ethosulfate; final concentration, 25 μmol/L) were added to each well containing 100 μl of culture medium. After 1.5 hours at 37°C, the optical density was read in a microplate spectrophotometer at a wavelength of 495 nm. The quantity of formazan product is directly proportional to the number of viable cells (6).

Immunocytochemistry

Transgene expression was determined by immunocyto-chemistry in J3T cells fixed 72 hours after infection with Ads (4% paraformaldehyde in phosphate-buffered saline, 15 min, at room temperature) and in dog striatal coronal sections (75 μm) cut with a microtome. Transgenes were detected with primary antibodies developed in our lab, diluted 1:1000, against βGal (41,48), against TK (1,2), and against Flt3L (1,2,15). Inflammatory cells were detected in dog striatal sections treated with trypsin and citrate buffer for antigen retrieval. Monoclonal primary

antibodies against dog CD3 ϵ (T cells) and CD18 (macrophages/activated microglia) were previously described (43). Antibody binding was revealed using 1:1000 dilutions of anti-rabbit biotinylated secondary antibodies (Dako, Carpinteria, CA), followed by incubation with peroxidase-conjugated avidin (Vector Laboratories, Burlingame, CA) and diaminobenzidine (Sigma Chemical Co., St. Louis, MO).

To detect in which cell types the transgenes are expressed, we combined β Gal, TK, and Flt3L primary antibodies with astrocytic and neuronal cell markers. Dog brain sections were blocked via incubation with 10% fetal calf serum and 0.3% Triton X100 from Fisher Scientific (Hanover Park, IL) for 1 hour before application of primary antibodies. A 1:500 dilution of guinea pig anti-glial fibrillar astrocytic protein (GFAP) (Advanced Immunochemical, Long Beach, CA) was used to detect astrocytes; and a 1:1000 dilution of mouse anti-NeuN antibody was used to label mature neuronal cells (Chemicon Inc., Temecula, CA). The fluorescent secondary antibodies used for detection were goat anti-rabbit Alexa 488, goat anti-guinea pig Alexa 647, and goat anti-mouse Alexa 594 (diluted 1:1000; Molecular Probes, Eugene, OR). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes/Invitrogen Corporation, Carlsbad, CA) and mounted with ProLong Antifade (Molecular Probes/Invitrogen Corporation).

Confocal Microscopy

Confocal micrographs were obtained using a Leica confocal microscope TCS SP2 with acousto-optical beam splitter equipped with a 405-nm violet diode ultraviolet laser, 488-nm Argon laser, 594-nm and 633-nm HeNe lasers, and using a HCX PL APO 63 $\times$ 1.4 numerical aperture oil objective (Leica Microsystems Heidelberg, Mannheim, Germany).

Statistical Analysis

Data were analyzed by two-way analysis of variance followed by Student-Newman-Keuls test. *P* values less than 0.05 were considered the cut-off point for significance.

RESULTS

In Vitro Gene Transfer into Dog Glioma Cells

To determine the feasibility of adenoviral-mediated gene transfer into dog GBM, we tested the transduction efficiency using Ads in canine glioma cells. J3T dog glioma cells were infected with Ads encoding the therapeutic transgenes HSV1-TK and Flt3L or the reporter gene β Gal. As shown in Figure 1, strong expression of β Gal, HSV1-TK, and Flt3L was detected by immunocytochemistry in J3T cells. Transduction efficiency was approximately 45% and similar for the three vectors (Fig. 1). We then tested whether or not the expressed trans-genes were biologically active because this is critical if the strategy is aimed at clinical implementation. β Gal enzymatic activity was readily detected in J3T cells protein extracts after infection with Ad- β Gal (Fig. 1A).

To test the bioactivity of Ad-TK, J3T cells were infected with Ad-TK for 72 hours, followed by incubation with the prodrug, GCV, for 72 hours. The cytotoxic effect of TK plus GCV was detected by the MTS viability assay. The incubation of cells with 10 μ mol/L of GCV in the absence of HSV1-TK expression had no cytotoxic effect per se. However, incubation with GCV after Ad-TK infection exerted a potent cytotoxic effect (Fig. 1B). As a control, we infected J3T cells with Ad- β Gal in the presence or absence of GCV. No cytotoxicity was observed in Ad- β Gal infected cells regardless of the presence or absence of GCV (not shown). Because Flt3L is secreted by the transduced cells, we determined its concentration in the supernatant of J3T cells infected with Ad-Flt3L using ELISA. The concentration of Flt3L in

the supernatant of these cultures reached levels of 450 µg/ml (10 µmol/L) 3 days after infection (Fig. 1C).

In Vivo Gene Transfer into the Brain of Dogs

Because adenoviral-mediated gene transfer to dog glioma cells was successful, we assessed Ad-mediated transgene expression into the dog brain. We administered Ads encoding βGal, HSV1-TK, or Flt3L using a dose of 8×10^7 pfu/5 µl saline per injection site into the cerebral cortex of beagle dogs. Blood was extracted the day of the surgery and the day of euthanasia to determine serum concentration of Flt3L and assess the presence of circulating antibodies to Ad. Cell type-specific transgene expression and infiltration of inflammatory cells in the brain were determined by immunocytochemistry. As shown in Figure 2, we detected expression of all transgenes, i.e., βGal, HSV1-TK, and Flt3L. The area transduced by the vectors was approximately 5 to 10 mm² around the injection sites. At the injection site, we detected the presence of inflammatory cells. Macrophages and activated microglia were detected through their immunoreactivity for CD18, and T cells were identified by CD3-positive immunoreactivity. Macrophage infiltration and activated microglia were detected in the site of infection (Fig. 2B). We also found T-cell infiltration, although to a lesser extent.

Cell type-specific expression was detected by double-labeling immunofluorescence using antibodies against GFAP and neuronal cell bodies (NeuN). Confocal analysis showed that βGal, HSV1-TK, and Flt3L are expressed in astrocytes (Fig. 3) and in neurons (Fig. 4). We determined that 75% of the transduced cells were astrocytes and 3% were neurons. The remaining 22% of the cells expressing the transgene were negative for GFAP or NeuN. The morphology of these cells was suggestive of oligodendrocytes.

Considering that Flt3L is a secreted cytokine, we determined the levels of Flt3L by ELISA in the serum from dogs that were bled before and 7 days after surgery. We detected no cross-reaction with dog endogenous Flt3L in the serum from non-injected dogs (data not shown). However, the concentration of Flt3L in the dog serum was 225 pg/ml 7 days after the injection of Ad-Flt3L in the brain.

Clinical Outcomes in Dogs Intracranially Injected with Ads

We monitored clinical parameters in the dogs before and after the intracranial administration of the Ads. We determined body temperature before the surgery and daily for the following 6 days. Dogs were bled the day of the surgery, 24 hours after surgery, and on the day of sacrifice, and blood counts and serum biochemistry were determined. Urine was collected by cystocentesis during the surgery and 3 days later. CSF was collected from the cisterna magna during the surgery and before sacrifice.

Body temperature measurements are shown in Figure 5. Three days after the surgery, the dog injected with Ad-TK and Ad-Flt3L experienced slight and transient hypothermia that resolved by 24 hours, and, toward the end of the study, at Day 6, the dog injected with Ad-βGal underwent mild hyperthermia.

All serum chemistry values were within or slightly outside the normal range at all time points, with the exception of the glucose on Day 0 (Table 1). However, the low glucose values are most likely caused by delayed separation of the serum from the cells, which quickly metabolizes the glucose. No abnormalities were detected in the urinalyses of any dog (not shown).

All complete blood count values are near or within normal range, except for a very mild lymphocytosis 24 hours after the surgery in the dog injected with Ad-βGal, and mild mature neutrophilia on the day of the surgery in the dog injected with Ad-TK and Ad-Flt3L (Table 2).

However, these values returned to normal by the next time point. No other abnormalities were noted in these animals. We did not detect circulating antibodies to Ad in the serum of these dogs.

The analysis of the CSF (Table 3) indicated that 7 days after the surgery, the cellularity of the samples was slightly increased, composed of few red blood cells along with a population of nucleated cells that included small and large mononuclear cells. Other parameters of the CSF, such as color, viscosity, and turbidity, remained normal.

DISCUSSION

Considering that dogs bearing spontaneous GBM constitute an excellent large GBM animal model, we tested the feasibility of adenoviral-mediated gene transfer of therapeutic transgenes to dog glioma cells (J3T) in vitro and into the brain of healthy dogs in vivo. In the present report, we show that J3T cells were efficiently transduced using Ad Type 5. The expression of β Gal, HSV-TK, and Flt3L was detected in these cells by immunocytochemistry. Transgene bioactivity was also assayed in J3T cells. β Gal activity was readily detected in cell protein extracts. HSV-TK cytotoxicity was observed only in the presence of the prodrug, GCV, which did not have a toxic effect per se. Because incubation with Ad- β Gal did not exert cytotoxic effects in the presence or absence of GCV, we can conclude that the Ad-TK cytotoxic effect is caused by GCV conversion by HSV-TK. Dog GBM cells were also capable of secreting Flt3L after infection with Ad-Flt3L, indicating that dog glioma cells are capable of expressing biologically active intracellular (β Gal and HSV1-TK) and secreted (Flt3L) transgenes. The concentration of Flt3L detected in the conditioned media from J3T dog glioma cells is in accordance with our previous results in murine and human glioma cells (5). J3T cells were previously shown to efficiently express transgenes delivered by Type 2 Ads, HSVs, and retroviral vectors (39). Our results demonstrate that dog glioblastomas will be susceptible to adenoviral Type 5-mediated gene transfer, as extensively shown in human GBM patients (13,19,22,23,26,28,38).

Delivery of the reporter gene was achieved in a brain tumor after intra-arterial administration of Ads to a dog bearing a benign meningioma (10). In a dog model of subarachnoid hemorrhage, administration of adenovirus Type 2 into the CSF was used for reporter gene transfer to cerebral blood vessels and meninges (33). However, Ads failed to reach the brain parenchyma after intracisternal administration, infecting brain arteries and ventricle ependymal lining (33). This constitutes the first report, to our knowledge, showing transgene expression in the dog brain parenchyma. Seven days after intracranial administration into the dog brain, β Gal, TK, and Flt3L were expressed in the dog brain, mainly in astrocytes. Moreover, serum Flt3L levels reached values of 225 pg/ml 7 days after the surgery in the dog that was injected with Ad-Flt3L in the brain. This data is in agreement with our previous findings in rats, in which intracranial injection of 5×10^7 infectious units Ad-Flt3L leads to serum levels of Flt3L of 200 pg/ml (15). Our results show that Ads are efficient gene transfer vehicles to deliver therapeutic genes into the dog brain, and that monitoring levels of expression of secreted transgene products in the serum will enable the assessment of secreted transgene expression within the brain in situ without the need to sacrifice animals.

In human GBM patients injected intratumorally with adenoviruses encoding p53, the expression of the therapeutic transgene was detected up to 5 mm from the needle tract (28). This gene therapy clinical trial proved efficacious in human GBM patients. Most clinical gene therapy protocols for GBM usually consist of the administration of several injections, ranging from 10 sites for conditionally replicative oncolytic viruses (13) to 50 to 70 injection sites for replication defective adenoviruses expressing therapeutic transgenes (28,40); this considerably increases the amount of tissue transduced by the therapeutic vectors. Our results in the dog

brain in vivo are, therefore, in line with what has been reported in humans. In addition, taking into account that Flt3L is a secreted cytokine that elicits a systemic anti-GBM immune response (2,15), and HSV-1 TK exhibits a strong bystander effect (5,32), only a limited number of cells need to be transduced for the therapy to succeed.

We detected mild infiltration of T cells and macrophages/ microglia in the brain parenchyma at the site of injection of all viruses. Accordingly, the analysis of the CSF only revealed a slight increase in cellularity 7 days after surgery. The slight increase in body temperature evidenced toward the end of the study, together with the mild mononuclear pleocytosis of the CSF and the infiltration of inflammatory cells, could be caused by the local acute innate immune response observed after adenovirus delivery into the brain (7). This local and transient inflammatory response is characterized by an increase in the levels of cytokines, i.e., tumor necrosis factor- α , interleukin-1, and interleukin-6 (7), as well as infiltration of inflammatory cells, such as macrophages, neutrophils, natural killer cells, and lymphocytes, as previously described in rodents (46,47). Importantly, we have previously demonstrated that this innate response does not adversely affect the levels of Ad-mediated transgene expression or the longevity of expression within the brain (31,46,47,54). These results suggest that adenoviral-mediated transgene delivery to the dog brain does not exert major adverse side effects. In agreement, clinical parameters, including serum biochemistry, blood counts, and body temperature were, overall, within the normal range. Although long-term studies would be necessary to determine chronic effects of adenovirus-mediated gene delivery to the brain, our results show that the intracranial administration of Ads is well tolerated and exerts no acute local or systemic side effects.

Although some humans exhibit preexisting Ad-neutralizing antibodies, it has been extensively demonstrated in human clinical trials that Ads successfully mediate therapeutic gene transfer and increased survival of GBM patients (28,40). Although we did not detect neutralizing antibodies in the dogs in this study, approximately 20% of the population of outbred dogs exhibit serum neutralizing antibodies against adenoviruses (52). Thus, canine spontaneous GBMs also mimic the human immune status against adenoviruses. In addition, several other factors make the spontaneous incidence of GBM in dogs an excellent opportunity to test novel anti-glioma therapeutic approaches prior to their implementation in human patients. The larger size of the dog brain would allow better optimization of doses and volumes used in gene therapy trials in human subjects. In addition, the ability to observe adverse side effects and behavioral abnormalities is easier in dogs than in rodent brain tumor models. Moreover, individual variations in outbred dogs will help to more closely mimic the clinical variability encountered in human patients. Considering that naturally occurring GBMs in dogs exhibit histological and clinical features that are similar to those of the human patients, and that the diagnosis can be performed by magnetic resonance imaging scans, GBM-bearing dogs constitute excellent candidates to test novel therapeutic approaches, including gene therapies. Moreover, this is a relatively frequent tumor in certain dogs, accounting for 12% of brain tumors, specially in brachycephalic breeds, such as Boston terriers and boxers, which are predisposed to develop GBM (4). In fact, the School of Veterinary Sciences, University of Minnesota, admits at least four dogs per month with a newly diagnosed brain tumor. Approximately 40 to 45% are diagnosed as GBM, on the basis of imaging studies. This suggests that an average of 18 to 20 dogs per year could be recruited for an efficacy clinical trial. In the present work, we show that J3T dog glioma cells can be readily infected using adenovirus Type 5 and that Ad5 administration in vivo into the dog brain leads to safe and efficient therapeutic gene transfer.

CONCLUSION

Ad Type 5 efficiently transduces dog glioma cells in vitro and the dog brain in vivo, exerting a low local acute inflammatory response without any further local or systemic adverse side

effects. Ad Type 5 constitutes an excellent gene therapy vector for a GBM efficacy trial in dogs as a prelude to human trials.

Acknowledgments

The work described in this article was funded by Grant 1RO1 NS44556.01 from the National Institute of Neurological Disorders and Stroke, by Grant 1 RO3 TW006273–01 from the National Institute of Diabetes and Digestive and Kidney to MGC, by Grants 1 RO1 NS42893, U54 4 NS04-5309, and R21 NS47298 from the National Institute of Neurological Disorders and Stroke (PRL); the Linda Tallen and David Paul Kane Annual Fellowship (MGC, PRL); the Bram and Elaine Goldsmith and the Medallions Group Endowed Chairs in Gene Therapeutics (PRL, MGC, respectively); and The Board of Governors at Cedars-Sinai Medical Center; grants from the Biomedical Engineering Institute (JRO), and Grant IRG-58-001-46-IRG35 from the American Cancer Society (JRO). We thank Dr. Shlomo Melmed for his academic leadership and Dr. Carlos Barcia for help and advice with confocal microscopy.

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FIGURE 1.

In vitro expression of β Gal (A), HSV1-TK (B), and Flt3L (C) in J3T dog glioma cells infected with Ads. J3T dog glioma cells were infected with 30 pfu/cell of Ad- β -Gal (A), HSV1-TK (Ad-TK, B), or Flt3L (Ad-Flt3L, C) under the control of the hCMV promoter. After 72 hours, J3T cell cultures were processed for immunocytochemistry (right panels), β Gal enzymatic activity, MTS viability assay, or Flt3L ELISA (left panels). Two-way analysis of variance were followed by Student-Newman-Keuls test. The percentage of cells expressing the transgenes are indicated at the top right-hand side of each image. Asterisk, $P < 0.05$.

FIGURE 2.

In vivo expression of β Gal (A), HSV-TK (B), and Flt3L (C), and inflammatory cell infiltration in the brain of dogs intracranially injected with Ads. Dogs were injected in the parietal neocortex with Ad- β Gal, Ad-TK, or Ad-Flt3L under the control of the hCMV promoter (8×10^7 pfu in 5 μ l per injection site). After 7 days, dogs were perfused/fixed and the brains removed and processed for immunocytochemistry. A, transgene expression was detected in 75- μ m coronal sections (original magnification, $\times 500$). B, macrophages/activated microglia were detected using an antibody to CD18 (original magnification, $\times 50$), and T cells were localized using an antibody to CD3 ϵ (original magnification, $\times 200$). Insets depict higher magnification images (original magnification, $\times 500$).

FIGURE 3.

In vivo expression of β Gal, HSV-TK, and Flt3L in astrocytes of dogs intracranially injected with Ads. Dogs were injected in the parietal neocortex with Ad- β Gal, Ad-TK, or Ad-Flt3L under the control of the hCMV promoter (8×10^7 pfu in 5 μ l per injection site). After 7 days, dogs were perfused/fixed, the brains removed, and transgene expression was detected in 75- μ m coronal sections. Transgene expression (green) was detected in astrocytes (GFAP, magenta) using indirect immunofluorescence. Nuclei were stained with DAPI (blue). Arrows indicate astrocyte processes exhibiting colocalization of GFAP and transgene (white).

FIGURE 4.

In vivo expression of β Gal, HSV-TK, and Flt3L in neurons of dogs intracranially injected with Ads. Dogs were injected in the parietal neocortex with Ad- β Gal, Ad-TK, or Ad-Flt3L under the control of the hCMV promoter (8×10^7 pfu in 5 μ l per injection site). After 7 days, dogs were perfused/fixed, the brains removed, and transgene expression was detected in 75- μ m coronal sections. Transgene expression (green) was detected in neurons (NeuN, red) using indirect immunofluorescence. Nuclei were stained with DAPI (blue). Arrows indicate neuron processes exhibiting colocalization of NeuN and transgene (orange).

FIGURE 5.

Body temperature in dogs intracranially injected with Ads. Dogs were injected in the parietal neocortex with Ad-βGal, Ad-TK, or Ad-Flt3L under the control of the hCMV promoter (8×10^7 pfu in 5 μ l per injection site). Body temperature was determined before the surgery and daily for the following 6 days.

TABLE 1

Serum chemistry profiles of dogs injected with adenoviruses^a

Parameter	Reference range	Ad-βGal		Ad-TK/Ad-Flt3L	
		Day 0	Day 1	Day 0	Day 1
Blood urea nitrogen (mg/dl)	9–31	13	14	18	13
Creatinine (mg/dl)	0.6–1.6	0.6	0.6	0.7	0.6
Calcium (mg/dl)	9.3–11.5	10.3	10.2	10.3	10.0
Phosphorus (mg/dl)	3.3–6.8	5.3	6.1	5.2	4.9
Magnesium (mg/dl)	1.7–2.4	1.9	1.7	1.7	1.6
Protein (mg/dl)	5.0–6.9	5.8	5.4	5.7	5.4
Albumin (mg/dl)	2.7–3.7	3.1	2.8	3.4	3.2
Globulin (mg/dl)	1.7–3.5	2.7	2.6	2.3	2.2
Sodium (mmol/L)	145–153	145	141	150	144
Chloride (mmol/L)	109–118	108	109	112	111
Potassium (mmol/L)	3.6–5.3	4.4	4.3	3.8	4.6
Bicarbonate (mmol/L)	15–25	22.0	23.0	20.0	24.0
Total bilirubin (mg/dl)	0–0.3	0.2	0.2	0.3	0.2
Alkaline phosphatase (U/l)	8–139	86	104	92	116
Gamma glutamyl transpeptidase (U/l)	0–6	<3	<3	<3	<3
Alanine transaminase (Uti)	22–92	48	48	98	147
Glucose (mg/dl)	75–117	42	108	46	96
Cholesterol (mg/dl)	143–373	171	160	159	160
Amylase (U/l)	275–1056	603	496	755	599

^a Ad-βGal, adenoviral vector encoding β-galactosidase; Ad-TK, adenoviral vector encoding thymidine kinase; Ad-Flt3L, adenoviral vector encoding Fms-like tyrosine kinase 3 ligand.

TABLE 2

Complete blood counts in dogs injected with adenoviruses^a

Parameter	Reference range	Ad-βGal			Ad-TK/Ad-FIt3L		
		Day 0	Day 1	Day 7	Day 0	Day 1	Day 7
White blood counts ($\times 10^3/\mu\text{l}$)	4.1–13.3	8.4	11.7	7.8	14.4	9.9	10.9
Segmented neutrophils ($\times 10^3/\mu\text{l}$)	2.1–11.2	5.88	6.90	6.01	11.38	5.54	5.34
Band neutrophils ($\times 10^3/\mu\text{l}$)	0–0.13	0.08	0.0	0.0	0.0	0.0	0.0
Lymphocytes ($\times 10^3/\mu\text{l}$)	0.78–3.36	1.34	3.63	1.33	2.16	2.77	4.25
Mononuclear cells ($\times 10^3/\mu\text{l}$)	0–1.2	0.84	0.70	0.47	0.86	0.40	0.87
Eosinophils ($\times 10^3/\mu\text{l}$)	0–1.2	0.25	0.47	0.0	0.0	1.19	0.44
Basophils ($\times 10^3/\mu\text{l}$)	0–0.13	0.0	0.0	0.0	0.0	0.0	0.0
Red blood cells ($\times 10^6/\mu\text{l}$)	5.71–88.29	6.59	6.06	6.83	6.13	6.01	6.15
Hemoglobin (g/dl)	13.5–19.9	15.1	13.9	15.5	14.6	14.1	14.6
Hematocrit (%)	38.5–56.7	44.0	40.0	45.1	41.2	40.8	41.6
Mean corpuscular volume (fl)	64–73	66.7	66.0	66.0	67.2	67.9	67.7
Mean corpuscular hemoglobin (pg)	21.8–26	22.9	23.0	22.7	23.8	23.5	23.8
Mean corpuscular hemoglobin concentration (g/dl)	33.6–36.6	34.4	34.8	34.4	35.4	34.7	35.2
Red cell distributive width (%)	12.5–16.5	14.3	14.9	15.2	15.2	15.6	15.5
Platelet counts ($\times 10^3/\mu\text{l}$)	160–425	199	183	224	314	326	381
Mean platelet volume (fl)	6–11	10.9	12.9	8.7	7.6	7.6	7.5
Platelet crit (%)	0.11–0.35	0.11	0.11	0.20	0.24	0.25	0.28
Platelet distribution width (%)	15.3–18.1	17.9	18.3	16.8	16.5	16.3	16.4
Thiamine pyrophosphate (g/dl)	5.8–7.2	6.1	6.0	6.2	5.9	5.9	5.7

^a Ad-βGal, adenoviral vector encoding β-galactosidase; Ad-TK, adenoviral vector encoding thymidine kinase; Ad-FIt3L, adenoviral vector encoding Fms-like tyrosine kinase 3 ligand.

TABLE 3

Cerebrospinal fluid analysis in dog injected with adenoviruses^a

Parameter	Reference range	Ad-βGal		Ad-Tk/Ad-Fit3L	
		Day 0	Day 7	Day 0	Day 7
Color	Colorless	Colorless	Colorless	Colorless	Colorless
Turbidity	Clear	Clear	Clear	Clear	Clear
Viscosity	Low	Low	Low	Low	Low
Volume (ml)	2.0	2.0	1.5	2.0	1.5
Nucleated cells/μL	<5	1	40	3	11
RBC/μL	<200	103	8	5	145
Diagnosis		Normal	Mild mononuclear pleocytosis	Normal	Mild mononuclear pleocytosis

^a Ad-βGal, adenoviral vector encoding β-galactosidase; Ad-Tk, adenoviral vector encoding thymidine kinase; Ad-Fit3L, adenoviral vector encoding Fms-like tyrosine kinase 3 ligand; RBC, red blood cells.