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Adenoviral Mediated Gene Transfer into the Dog 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 (HSV1-TK) and immuno-stimulatory (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-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 pfu/cell, 72 h) encoding β-Gal (Ad-βGal), TK (Ad-TK) or Flt3L (Ad-Flt3L). We determined transgene expression by immunocytochemistry, βGal activity, Flt3L ELISA and TK-induced cell death. Ad vectors 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 βgal was detected in dog glioma cells *in vitro* (45% trasduction efficiency) and in the dog brain *in vivo* (10mm^2 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. *Conclusions.* 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 trails.

Running Title: *In vivo* Gene transfer into canine brain

Keywords: adenovirus, dog, ganciclovir, gene therapy, glioblastoma, Flt3L, HSV1-TK.

INTRODUCTION

GBM, the most common type of primary brain tumor in adults, is a very aggressive and locally invasive tumor. Survival of patients affected by GBM has remained virtually unchanged during the last decades (6-12 months post-diagnosis) despite advances in surgery, radiation and chemotherapy (8, 12, 16, 24, 27, 37). Considering the very poor prognosis of patients with high grade glioma, novel therapeutic approaches are needed. Gene therapies are currently being developed and implemented in rodent pre-clinical 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 pre-clinical rodent experiments have led to human clinical trials, some of which showed therapeutic efficacy (13, 26, 40, 50). Nevertheless, the lack of a pre-clinical model that closely mimics the human condition and neuro-anatomy, 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 immuno-stimulatory therapy in an large, intracranial syngenic GBM model in Lewis rats in which the tumor occupies most of the volume of the striatum in one hemisphere, using adenoviral-mediated gene transfer of HSV1-TK and Fms-like tyrosine kinase 3 ligand (Flt3L) (1, 2). Since one of our aims is to translate this approach to human patients, we first aim to implement it in dogs with spontaneous GBM, which may better model human disease parameters.

Dogs have large brains (72 g in Beagles) compared to rodents. This makes them an attractive model to test gene delivery efficiency, evaluate vector and transgene biodistribution, and optimize treatment protocols prior to 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 very similar. Also, there is a high correlation of neuroimaging features seen with MRI in canine and human GBM, which is used as a diagnostic tool for dog GBM. Peritumoral edema is a consistent finding in both human and canine GBM (30, 35).

Canine GBMs are histopathologically indistinguishable from human GBM (30). Both are highly infiltrative, contain areas of intratumoral necrosis, pseudopalisading, hemorrhages, 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 paper, we demonstrate that adenoviral (Ad) vectors 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 herpes simplex virus 1 derived thymidine kinase (HSV1-TK) and *fms-like tyrosine kinase 3 ligand* (Flt3L), or the reporter gene, β-Galactosidase.

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 very efficient in eliminating large, intracranial GBM in the syngenic Lewis rat model where all other therapies fail (2). We administered Ad vectors *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 neuro-pathological 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.

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MATERIALS AND METHODS

First generation adenoviral vectors (Ads)

The first-generation replication-defective recombinant adenovirus type 5 vectors (Ad) expressed β-Galactosidase (β-Gal) (Ad-βGal), 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 an MOI of 3 pfu/cell of vector seed stock. The cells were harvested 48 hours later, lysed with 5% deoxycholate and DNaseI, and Ad vectors were purified by ultracentrifugation over two cesium chloride step gradients (36, 42). Vectors were titered in triplicate by end-point dilution, cytopathic effect assay (42). The titers were as follows: Ad-hCMV-βgal (RAd35) (Ad-βGal 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 (RCA) (18, 42) and for LPS contamination (Cambrex, East Rutherford, NJ) (14, 42). Virus preparations used were free from RCA and LPS contamination. The expression of the transgenes encoded within the first generation and high capacity adenoviral vectors was verified by infecting COS-7 cells with an MOI of 100 vp./cell and performing immunocytochemistry with antibodies for βgal, TK and Flt3L as described below. Circulating anti-Ad antibodies were determined as described previously (52).

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 DMEM medium supplemented with 10% (vol /vol) fetal calf serum (FCS), 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine, and 2 mM non-essential aminoacids. Cells were infected with a dose of 30 plaque forming units (pfu)/cell of Ad-TK, Ad-Flt3L or, as a control, Ad-βGal under the

control of the human cytomegalovirus (hCMV) promoter. After 72 h, cells were processed for immunocytochemistry, MTS viability assay or β-Gal enzymatic activity assay, and the supernatant were collected for Flt3L 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 (IACUC) protocol and every effort was made to minimize pain and discomfort. The housing area was maintained at 12 hrs light and 12 hrs. dark at 22 ± 2 °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 h after surgery and the day of euthanasia. Rectal body temperature was monitored daily during the duration of the study.

Anesthesia and Surgery/Gene Delivery

Before the surgery, the dogs were sedated with acepromazine (0.01 mg/kg, IM). An intravenous catheter was placed in a cephalic vein and anesthesia was induced with sodium thiopental (5 mg/kg, IV). 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 Ringers solution (10 ml/kg/hr) was administered IV during anesthesia. Carprofen, a non-steroidal anti-inflammatory drug, (2.2 mg/kg PO every 12 hours) and buprenorphine (0.01 mg/kg IV and SC every 4-6 hours as needed) were used for postoperative analgesia.

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) 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 were diluted with sterile saline to 8×10^8 plaque forming units (pfu) in a final volume of 50 µl. Five bilateral injections were given; spaced 1 cm apart at a depth of 2 cm (from cortical surface) in 5µl/site (8×10^7 pfu/injection site) over 5min. 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 (PDS) 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) in interrupted cruciate sutures.

Monitoring and Euthanasia of Dogs

Body temperature was taken with a rectal thermometer. Blood and CSF, from the cisternae magna, were collected immediately before surgery and three days afterwards. Complete blood count (CBC), 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 sodium pentobarbital (200 mg/kg). After euthanasia, all dogs were perfused with freshly prepared tyrode's solution (132 mM NaCl/1.8 mM $CaCl_2$/ 0.32 mM NaH_2PO_4/5.56 mM glucose/11.6 mM $NaHCO_3$/2.68 mM KCL) with 500 units of heparin with O^2 aeration by cardiac perfusion followed by perfusion with 4% paraformaldehyde. Cerebral hemispheres were removed and fixed in 4% paraformaldehyde in PBS for 5 days.

β-galactosidase activity

J3T dog glioma cells (5×10^4/well) were infected with 30 pfu/cell of Ad-βGal. After 72 h protein extracts were prepared by three freeze-thaw cycles in PBS containing a cocktail of protease inhibitors (Endogen). β-galactosidase activity was measured by conversion of o-nitrophenyl-β-D-galactopyranoside in 10 mM MgCl2/0.45 M/2-mercaptoethanol, which was detected at 420 nm absorbance. β-

galactosidase activity was expressed as enzymatic activity/min ([*o*-nitrophenol (mg/ml)]/(time (min) x [protein (mg/ml)]).

Flt3L ELISA

J3T dog glioma cells (5×10^4/well) were infected with 30 pfu/cell of Ad-Flt3L. After 72 h, the supernatant was collected, cellular debris were removed by centrifugation and stored at -70°C. Flt3L concentration was determined in 50 µl of J3T cell culture supernatant and in dog serum before and seven days after adenoviral administration using a commercial ELISA kit (21-377-296, R&D systems, MN, USA) 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 h, the cells were incubated for additional 72 h in the presence of 10 µM of the prodrug ganciclovir (GCV). Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H

tetrazolium (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 µm) were added to each well containing 100 µl of culture medium. After 1.5 h at 37 C, the OD 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 immunocytochemistry in J3T cells fixed 72 h after infection with Ads (4% parafolmadehyde in PBS, 15 min., at room temperature) and in dog striatal coronal sections (75µm) cut with a microtome. Transgenes were detected with anti-βgal (41, 48), anti-TK (1, 2), and anti-Flt3L (1, 2, 15) specific primary antibodies (1:1000) developed in our lab. Inflammatory cells were detected in dog striatal sections treated with trypsin and citrate buffer for antigen retrieval. Monoclonal primary antibodies against dog CD3ε (T cells) and CD18 (macrophages/activated microglia) were described previously [43]. Antibody binding was revealed using anti-rabbit biotinylated secondary

antibodies (DAKO 1:1000), followed by incubation with peroxidase-conjugated avidin (Vector) and diaminobenzidine (SIGMA).

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 with 10% fetal calf serum and 0.3% Triton X100 from Fisher Scientific (Hanover Park, IL) for one hour incubation before application of primary antibodies. Guinea pig anti-glial fibrillar astrocytic protein (GFAP) (Advanced Immunochemical, Long Beach, CA) (1:500) was used to detect astrocytes and mouse anti-NeuN antibody was used to label mature neuronal cell (Chemicon Inc., Temecula, CA) (1:1000). The fluorescent secondary antibodies (1:1000) used for detection were goat anti-rabbit Alexa 488, goat-anti-guinea pig Alexa 647 and goat anti-mouse Alexa 594 (Molecular Probes, Eugene, OR). Nuclei were stained with DAPI (Molecular Probes/Invitrogen Corporation, Carlsbad, CA, USA) and mounted with ProLong Antifade (Molecular Probes/Invitrogen Corporation, Carlsbad, CA, USA).

Confocal microscopy

Confocal micrographs were obtained using a Leica confocal microscope TCS SP2 with AOBS equipped with a 405 nm violet diode UV laser, 488 nm Argon laser, 594, and 633 nm HeNe lasers, and using a HCX PL APO 63x 1.4 N.A. oil objective (Leica Microsystems Heidelberg, Mannheim, Germany).

Statistical analysis

Data were analyzed by two-way ANOVA followed by Student-Newman-Keuls test. $p < 0.05$ was considered the cut off point for significance.

RESULTS

In vitro gene transfer into dog glioma cells

In order to determine the feasibility of adenoviral mediated gene transfer into dog GBM, we tested the transduction efficiency using Ad vectors 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 around 45% and similar for the three vectors (Fig 1). We then tested whether the expressed transgenes were biologically active as this is critical if the strategy is aimed at clinical implementation. β-Galactosidase enzymatic activity was readily detected in J3T cells protein extracts after infection with Ad-β-Gal (Fig 1 A).

To test the bioactivity of Ad-TK, J3T cells were infected with Ad-TK for 72 h, followed by the incubation with the prodrug GCV for 72 h. The cytotoxic effect of TK/GCV was detected by the MTS viability assay. The incubation of cells with 10 µM 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 1 B). As a control, we infected J3T cells with Ad-βGal 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). Since Flt3L is secreted by the transduced cells, we determined its concentration in the supernatant of J3T cells infected with Ad-Flt3L using an ELISA. The concentration of Flt3L in the supernatant of these cultures reached levels of 450 µg/ml (10 µM) 3 days after infection (Fig 1 C).

In vivo gene transfer into the brain of dogs

Since adenoviral-mediated gene transfer to dog glioma cells was successful, we assessed Ad mediated transgene expression into the dog brain. We administered Ads encoding β-Gal, HSV1-TK or Flt3L using a dose of $8x10^7$ plaque forming units (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 anti-Ad antibodies. Cell type-specific transgene expression and infiltration of inflammatory cells in the brain was 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 around 5-10 mm^2 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 anti-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 cells infiltration, although to a lesser extent.

Cell type-specific expression was detected by double labeling immunofluorescence using antibodies against glial fibrilary astrocytic protein (GFAP) and neuronal cell body (NeuN). Confocal analysis showed that βGal, HSV1-TK and Flt3L are expressed in astrocytes (Fig. 3) and in neurons (Figure 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 which were bled prior 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 adenovirus vectors

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

Body temperature measurements are shown in Figure 5. The dog injected with Ad-TK and Ad-Flt3L experienced slight and transient hypothermia 3 days after the surgery that resolved by 24 h, while the dog injected with Ad-β-Gal underwent mild hyperthermia towards the end of the study, at day 6.

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 I). However, the low glucose values are most likely due to 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 present in the dog injected with Ad-β-Gal 24 h after the

surgery and mild mature neutrophilia in the dog injected with Ad-TK and Ad-Flt3L the day of the surgery (Table II). However, these values returned to normal by the next time point. No other abnormalities were noted in these animals. We did not detect anti-Ad circulating antibodies in the serum of these dogs.

The analysis of the CSF (Table III) indicated that 7 days after the surgery the cellularity of the samples was slightly increased, composed of very 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 βGal, HSV-TK and Flt3L was detected in these cells by immunocytochemistry. Transgene bioactivity was also assayed in J3T cells. βGal activity was readily detected in cell protein extracts. HSV-TK cytotoxicity was observed only in the presence of the prodrug ganciclovir, which did not have a toxic effect *per se*. Since incubation with Ad-βGal did not exert cytotoxic effects in the presence or absence of GCV, we can conclude that Ad-TK cytotoxic effect is due to 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 (βGal and HSV1-TK) and secreted (Flt3L) transgenes. The

concentration of Flt3L detected in the conditioned media from dog J3T 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 Ad type 2 vectors, herpes simplex viruses 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 reporter gene was achieved in a brain tumor after intraraterial 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 arterias and ventricle ependymal lyning (33). This constitutes the first report showing transgene expression in the dog brain parenchyma. Seven days after intracranial administration into the dog brain, βGal, 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 which 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 adenoviral vectors 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 in the administration of several injections, ranging from 10 sites for conditionally replicative oncolytic viruses (13) to 50-70 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. Also, taking into account that Flt3L is a secreted cytokine which elicits a systemic anti-GBM immune response [Ali et al. 2005; Curtin et al. 2006], and HSV-1 TK exhibits an strong by-stander effect [Candolfi et al. 2006; Maleniak et al. 2001; Windeatt et al. 2000], 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 towards the end of the study, together with the mild mononuclear pleocytosis of the CFS and the infiltration of inflammatory cells could be due to 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., TNF-α, IL-1 and IL-6 (7), as well as infiltration of inflammatory cells, such as macrophages, neutrophils, NK 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, 53). 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 intracranial administration of adenoviral vectors is well tolerated and exert no acute local or systemic side effects.

Although some humans exhibit pre-existing anti-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, around 20% of the population of outbred dogs exhibit serum neutralizing antibodies against adenoviruses (51). 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 as a prelude 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 adverse side effects and behavioral abnormalities is easier in dogs than in rodent brain tumor models. Moreover, individual variations in outbreed dogs will help to mimic closer the

clinical variability encountered in human patients. Considering that naturally occurring GBMs in dogs exhibit histological and clinical features that are very similar to those of the human patients, and that the diagnosis can be performed by MRI, GBM bearing dogs constitute excellent candidates to test novel therapeutic approaches, including gene therapies. <u>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 40-45% are diagnosed as GBM, based on imaging studies. This suggests that an average of 18-20 dogs per year could be recruited for an efficacy clinical trial.</u> 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.

CONCLUSIONS

Adenoviral vectors type 5 efficiently transduce 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. Ad5 constitutes an excellent gene therapy vector for a GBM efficacy trial in dogs as a prelude to human trials.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. *In vitro* **expression of β-Gal (A), HSV1-TK (B) and Flt3L (C) in J3T dog glioma cells infected with adenoviral vectors.** J3T dog glioma cells were infected with 30 pfu/cell of adenoviruses encoding β-Galactosidase (Ad-βGal, **A**), HSV1-TK (Ad-TK, **B**), or Flt3L (Ad-Flt3L, **C**) under the control of the human CMV promoter. After 72 h, J3T cell cultures were processed for immunocytochemistry (*right panels*), βGal enzymatic activity, MTS viability assay, or Flt3L ELISA (*left panels*). * $p < 0.05$. Two-way ANOVA followed by Student-Newman-Keuls test. The percentage of cells expressing the transgenes are indicated at the top right hand side of each image.

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 adenoviral vectors.** Dogs were injected in the parietal neocortex with Ad-βGal, Ad-TK or Ad-Flt3L under the control of the human CMV promoter (8×10^7 pfu in 5ul 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 μm coronal sections. **B**, Macrophages/activated microglia were

detected using an anti-CD18 antibody and T cells were localized using an anti-CD3ε antibody. Insets depict higher magnification pictures.

Figure 3. *In vivo* **expression of β-Gal, HSV-TK and Flt3L in astrocytes of dogs intracranially injected with adenoviral vectors.** Dogs were injected in the parietal neocortex with Ad-βGal, Ad-TK or Ad-Flt3L under the control of the human CMV promoter ($8x10^7$ pfu in 5ul per injection site). After 7 days, dogs were perfused/fixed, the brain removed and transgene expression was detected in 75 μm coronal sections. Transgene expression (green) was detected in astrocytes (GFAP, magenta) using indirect immunofluorescence. Nuclei were stained with DAPI (blue). Arrows indicate astrocytes processes exhibiting colocalization of GFAP and transgene (white).

Figure 4. *In vivo* **expression of βGal, HSV-TK and Flt3L in neurons of dogs intracranially injected with adenoviral vectors.** Dogs were injected in the parietal neocortex with Ad-βGal, Ad-TK or Ad-Flt3L under the control of the hCMV promoter ($8x10^7$ pfu in 5ul per injection site). After 7 days, dogs were perfused/fixed, the brain was removed and transgene expression was detected in 75 μm coronal sections. Transgene expression (green) was detected in neurons (NeuN, red) using indirect immunofluorescence. Nuclei were stained with DAPI (blue). Arrows indicate neurons processes exhibiting colocalization of NeuN and transgene (orange).

Figure 5. Body temperature in dogs intracranially injected with adenoviral vectors. 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 5ul per injection site). Body temperature was determined prior the surgery and daily for the following 6 days.
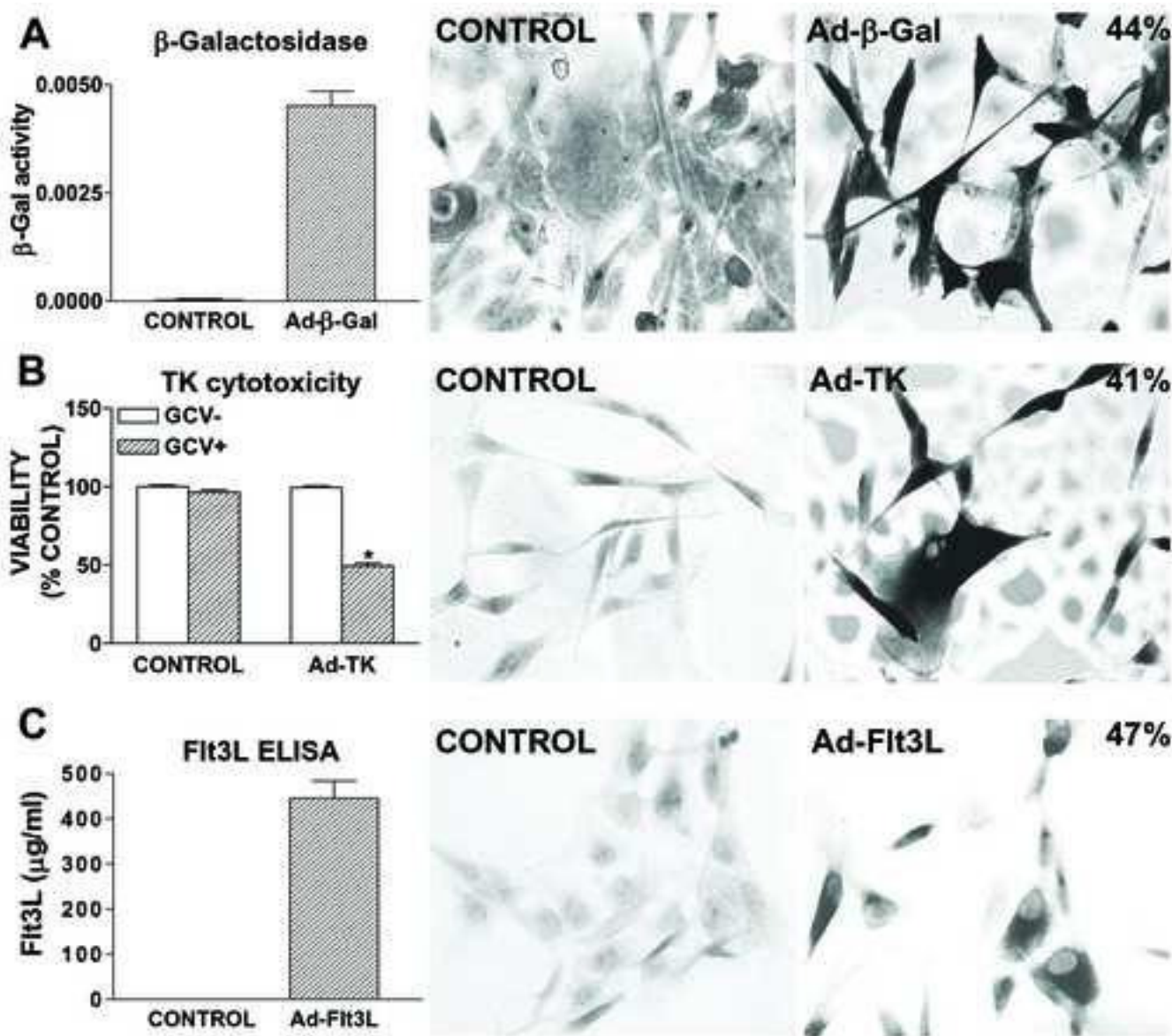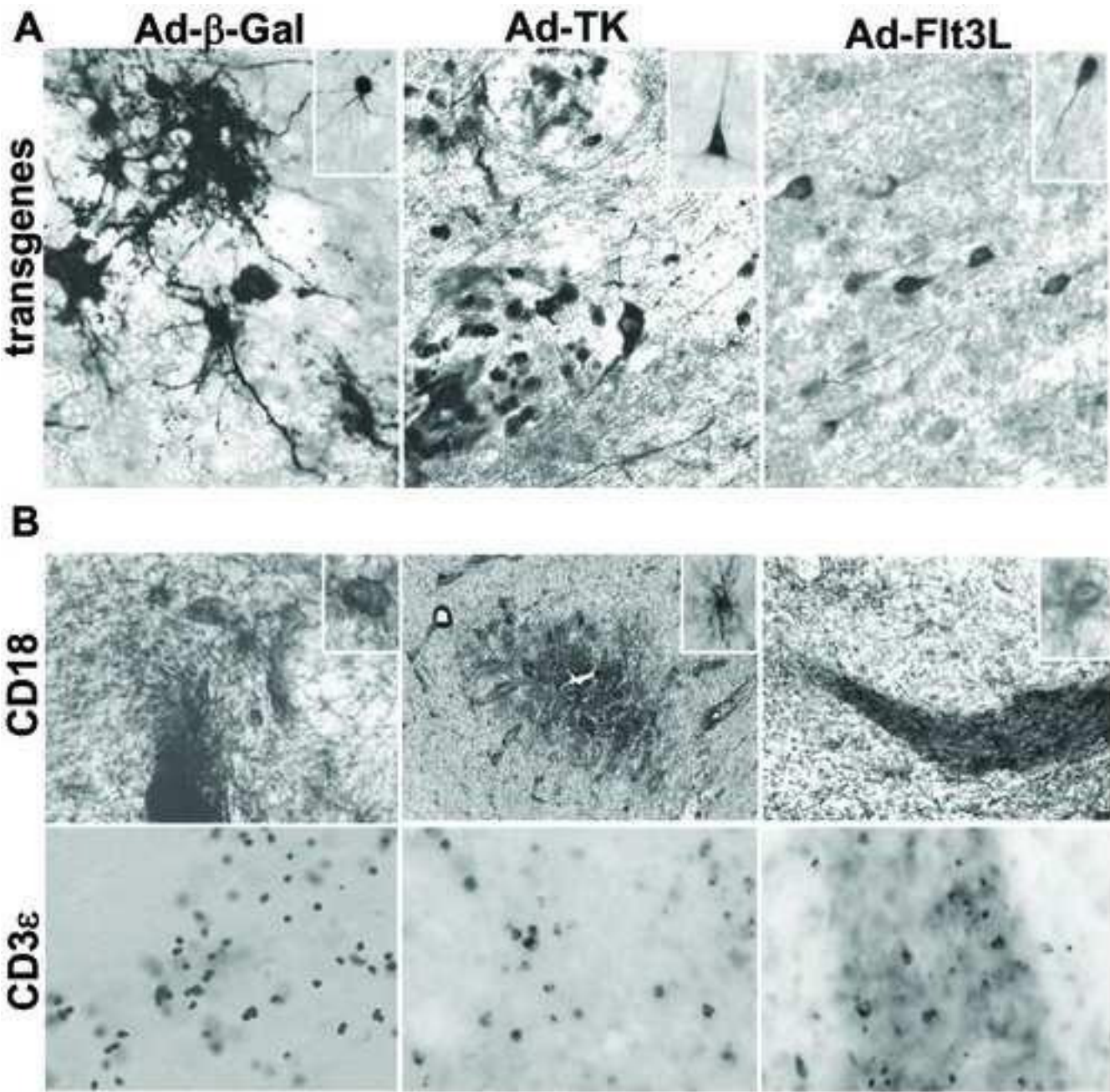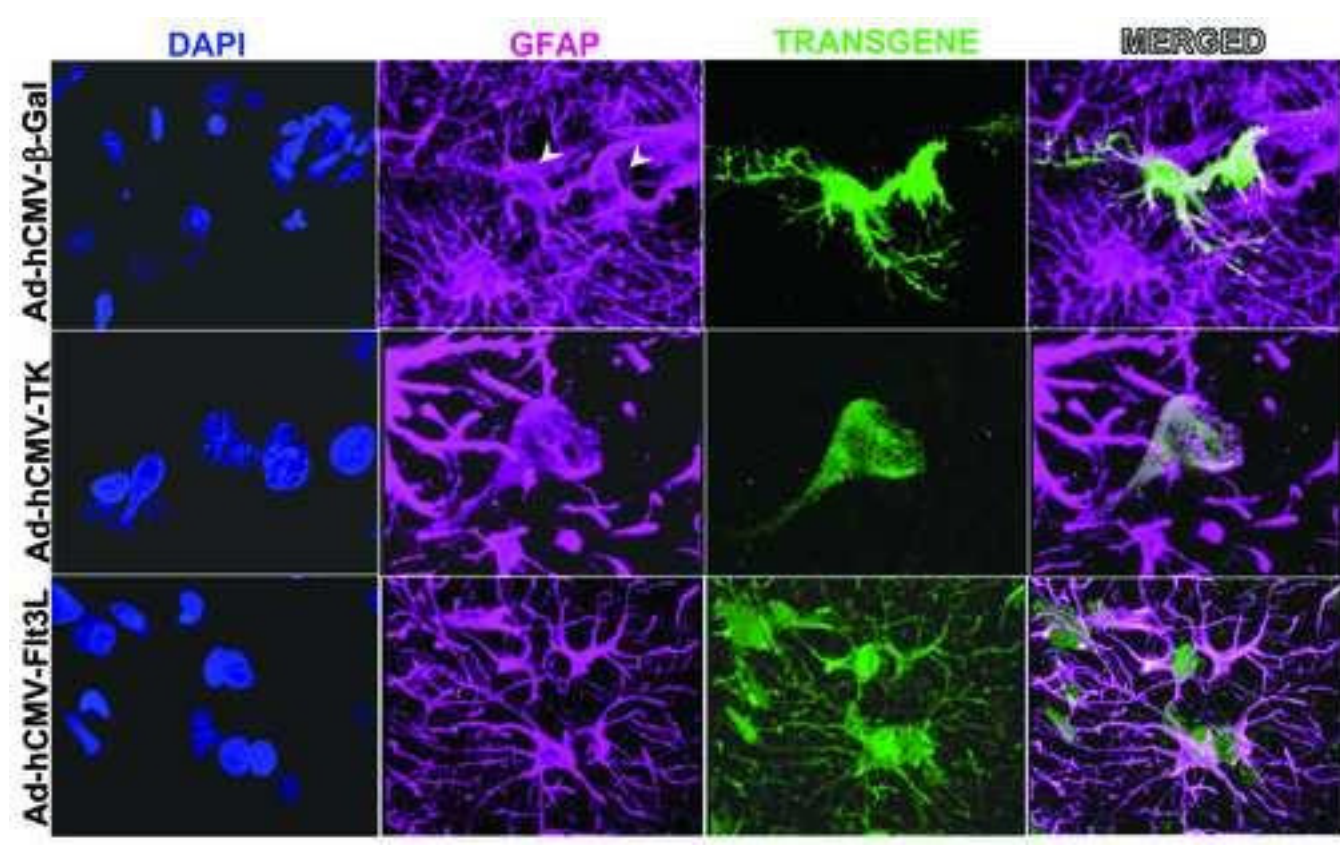
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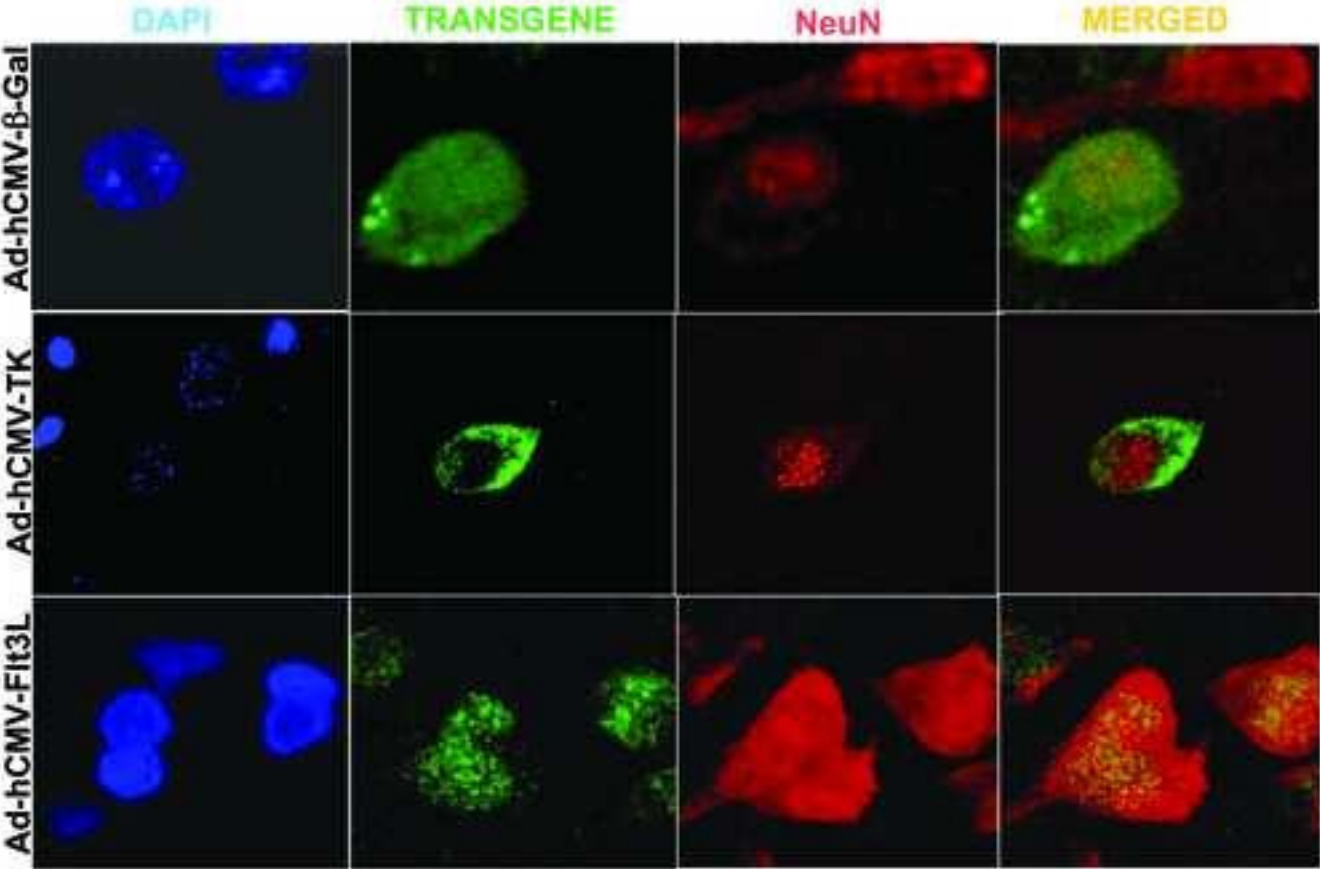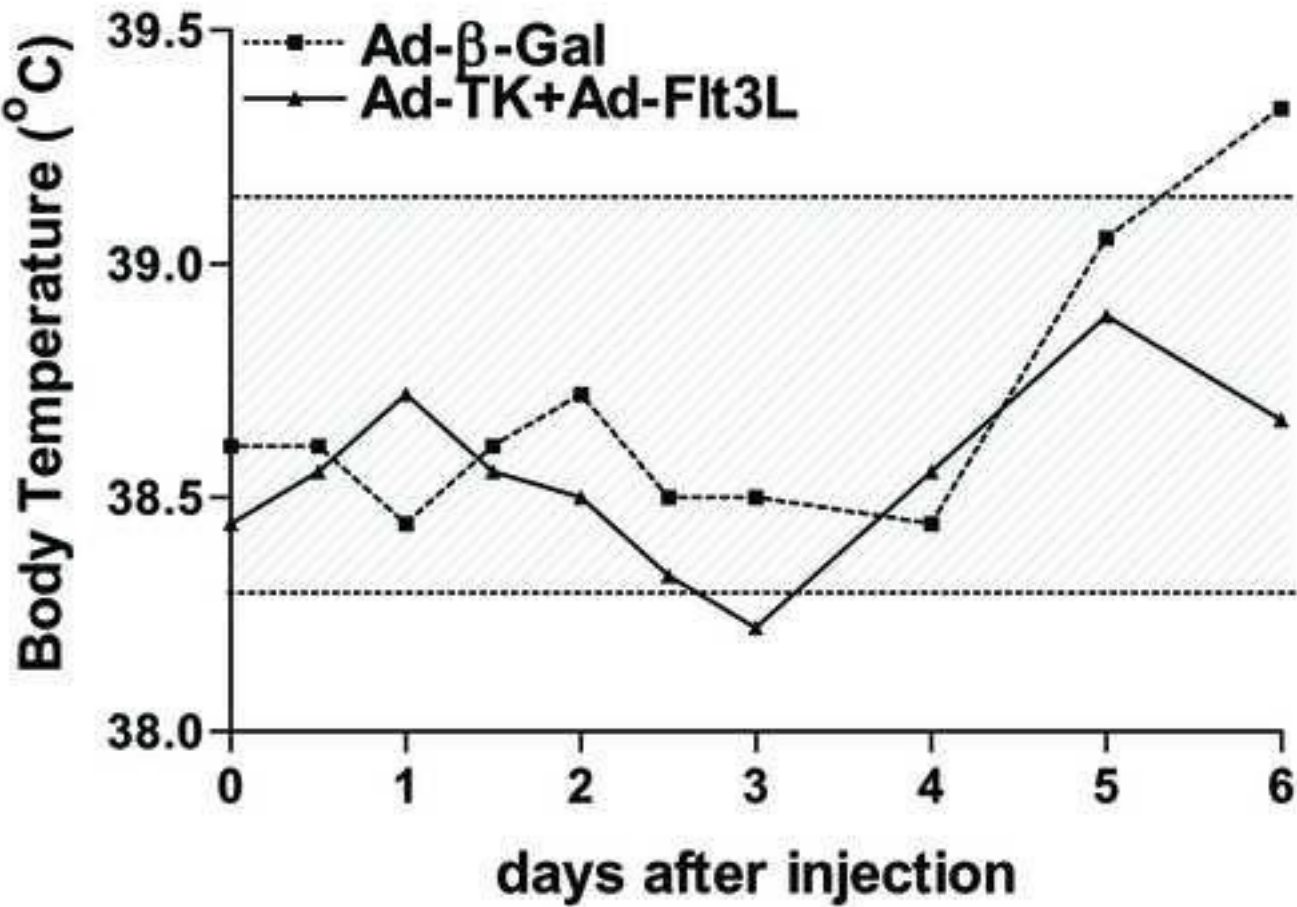
Table I. Serum chemistry profiles of dogs injected with adenoviruses

Parameter	Ref range	Ad-β-Gal			Ad-TK/Ad-Flt3L		
		day 0	day 1	day 7	day 0	day 1	day 7
Blood urea nitrogen (mg/dl)	9-31	13	14	16	18	13	12
Creatinine (mg/dl)	0.6-1.6	0.6	0.6	0.7	0.7	0.6	0.7
Calcium (mg/dl)	9.3-11.5	10.3	10.2	10.7	10.3	10.0	10.4
Phosporus (mg/dl)	3.3-6.8	5.3	6.1	6.0	5.2	4.9	5.4
Magnesium (mg/dl)	1.7-2.4	1.9	1.7	1.8	1.7	1.6	1.6
Protein (mg/dl)	5.0-6.9	5.8	5.4	5.8	5.7	5.4	5.3
Albumin (mg/dl)	2.7-3.7	3.1	2.8	3.3	3.4	3.2	3.3
Globulin (mg/dl)	1.7-3.5	2.7	2.6	2.5	2.3	2.2	2.0
Sodium (mmol/L)	145-153	145	141	146	150	144	145
Chloride (mmol/L)	109-118	108	109	112	112	111	111
Potassium (mmol/L)	3.6-5.3	4.4	4.3	4.6	3.8	4.6	4.5
Bicarbonate (mmol/L)	15-25	22.0	23.0	23.0	20.0	24.0	24.0
Total bilirubin (mg/dl)	0-0.3	0.2	0.2	0.2	0.3	0.2	0.2
Alkaline phosphatase (U/l)	8-139	86	104	93	92	116	92
Gamma glutamyl transpeptidase (U/l)	0-6	<3	<3	<3	<3	<3	<3
Alanine transaminase (U/l)	22-92	48	48	28	98	147	93
Glucose (mg/dl)	75-117	42	108	100	46	96	113
Cholesterol (mg/dl)	143-373	171	160	185	159	160	168
Amylase (U/l)	275-1056	603	496	486	755	599	745

Table (NO EXCEL FILES! MUST BE MS WORD FILE!)

Table II. Complete blood counts in dogs injected with adenoviruses

Parameter	Ref range	Ad-β-Gal			Ad-TK/Ad-Flt3L		
		day 0	day 1	day 7	day 0	day 1	day 7
White blood counts (x 10^3/ul)	4.1-13.3	8.4	11.7	7.8	14.4	9.9	10.9
Segmented neutrophils (x10^3/ul)	2.1-11.2	5.88	6.90	6.01	11.38	5.54	5.34
Band neutrophils (x 10^3/ul)	0-0.13	0.08	0.0	0.0	0.0	0.0	0.0
Lymphocytes (x 10^3/ul)	0.78-3.36	1.34	3.63	1.33	2.16	2.77	4.25
Mononuclear cells (x 10^3/ul)	0-1.2	0.84	0.70	0.47	0.86	0.40	0.87
Eosinophils (x 10^3/ul)	0-1.2	0.25	0.47	0.0	0.0	1.19	0.44
Basophils (x 10^3/ul)	0-0.13	0.0	0.0	0.0	0.0	0.0	0.0
Red blood cells (x 10^6/ul)	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 (MCV, fl)	64-73	66.7	66.0	66.0	67.2	67.9	67.7
Mean corp hemoglobin (pg)	21.8-26	22.9	23.0	22.7	23.8	23.5	23.8
Mean corp hemog concent (MCHC, g/dl)	33.6-36.6	34.4	34.8	34.4	35.4	34.7	35.2
Red cell distributive width (RDW, %)	12.5-16.5	14.3	14.9	15.2	15.2	15.6	15.5
Platelet counts (x 10^3/ul)	160-425	199	183	224	314	326	381
Mean platelet volume (MPV, fl)	6-11	10.9	12.9	8.7	7.6	7.6	7.5
Platelet crit (PCT, %)	0.11-0.35	0.11	0.11	0.20	0.24	0.25	0.28
Platelet distribution width (PDW, %)	15.3-18.1	17.9	18.3	16.8	16.5	16.3	16.4
Thiamine pyrophosphate (TPP, g/dl)	5.8-7.2	6.1	6.0	6.2	5.9	5.9	5.7

Table (NO EXCEL FILES! MUST BE MS WORD FILE!)

Table III. Cerebrospinal fluid analysis in dog injected with adenoviruses

Parameter	Ref range	Ad-β-Gal		Ad-TK/Ad-Flt3L	
		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	1.5	2.0	1.5
Nucleated cells/uL	<5	1	40	3	11
RBC/uL	<200	103	8	5	145
Diagnosis		normal	mild mononuclear pleocytosis	normal	mild mononuclear pleocytosis

RBC: red blood cells