Safety Profile of Gutless Adenovirus Vectors Delivered into the Normal Brain Parenchyma: Implications for a Glioma Phase 1 Clinical Trial

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Abstract

Adenoviral vectors (Ads) have been evaluated in clinical trials for glioma. However, systemic immunity against the vectors can hamper therapeutic efficacy. We demonstrated that combined immunostimulation and cytotoxic gene therapy provides long-term survival in preclinical glioma models. Because helper-dependent high-capacity Ads (HC-Ads) elicit sustained transgene expression, in the presence of antiadenoviral immunity, we engineered HC-Ads encoding conditional cytotoxic herpes simplex type 1 thymidine kinase and immunostimulatory cytokine Fms-like tyrosine kinase ligand-3 under the control of the TetOn system. Escalating doses of combined HC-Ads (10^8, 10^9, and 10^10 viral particles [VP]) were delivered into the rat brain. We assessed neuropathology, biodistribution, transgene expression, systemic toxicity, and behavioral impact at acute and chronic time points after vector delivery. Histopathological analysis did not reveal any evidence of toxicity or long-term inflammation at the lower doses tested. Vector genomes were restricted to the injection site. Serum chemistry did not uncover adverse systemic side effects at any of the doses tested. Taken together, our data indicate that doses of up to 10^9 VP of each HC-Ad can be safely administered into the normal brain. This comprehensive toxicity and biodistribution study will lay the foundations for implementation of a phase 1 clinical trial for GBM using HC-Ads.

Introduction

Despite advances in neurosurgical techniques, radiotherapy, and chemotherapy, the prognosis in glioblastoma multiforme (GBM) remains poor, with median survival of 14.6–19.6 months (Stupp et al., 2005; Grossman et al., 2010). GBM is the most common primary brain tumor in adults, and its highly infiltrative nature precludes complete surgical resection; thus, the tumor almost invariably recurs. Thus, immunotherapies are attractive adjuvants for this devastating disease.

We have developed a combined, conditional cytotoxic/immune stimulatory gene therapy strategy to treat GBM (Ali et al., 2005; Candolfi et al., 2009; Curtin et al., 2009; Ghulam Muhammad et al., 2009). This involves treatment with two genetically engineered first-generation adenoviral vectors (Ads) to deliver the conditionally cytotoxic herpes simplex type 1 (HSV1) thymidine kinase (TK) that kills proliferating tumor cells in the presence of the prodrug ganciclovir (GCV) (Dewey et al., 1999) and human soluble Fms-like tyrosine kinase ligand-3 (Flt3L), which recruits bone marrow-derived antigen-presenting cells into the brain tumor microenvironment (Ali et al., 2004; Curtin et al., 2006). This strategy has been shown to be highly effective in several syngeneic preclinical rodent GBM models, that is, rats bearing intracranial CNS-1, 9L, and F98 tumors (Ali et al., 2005; Ghulam
Muhammad et al., 2009) and mice bearing intracranial GL26, GL261, and B16-F10 tumors (Candolfi et al., 2009; Curtin et al., 2009). In addition, the treatment with Ad-TK plus Ad-Fli3L induces GBM-specific immunological memory that is effective in treating intracranial multifocal and peripheral models of GBM (King et al., 2008a,b; Ghulam Muhammad et al., 2009). This combined gene therapy strategy received approval from the U.S. Food and Drug Administration (FDA) for a phase 1 clinical trial for GBM (BB-IND 14574; NIH/OBA Protocol # 0907-990; OSU Protocol # 10089).

With the aim to improve the gene delivery platform to achieve regulatable Fli3L expression and elude antiadeno-virus immune responses, which could curtail therapeutic transgene expression, we have developed helper-dependent high-capacity adenoviral (HC-Ad) vectors expressing HSV1-TK under the control of a constitutive promoter and human soluble Fli3L (hsFli3L) under the control of the tightly regulatable mCMV-TetOn expression system (Xiong et al., 2006; Curtin et al., 2008). Using these HC-Ad vectors, we demonstrated their therapeutic efficacy and high safety profile after delivery into a large tumor mass in a syngeneic, orthotopic rat model of GBM (Muhammad et al., 2010; Puntel et al., 2010b).

In the present study, as a prelude to a novel phase 1 clinical trial for GBM, we conducted a dose-escalation and biodistribution study of HC-Ad-TetOn-Fli3L, plus HC-Ad-TK when delivered into the naive rat brain to determine the maximal tolerated dose (MTD) of these vectors. After injection of \(1 \times 10^9\), \(1 \times 10^3\), or \(1 \times 10^5\) viral particles (VP) of each HC-Ad, assessment of brain tissue integrity, immune infiltrates, vector biodistribution and transgene expression, as well as putative systemic and neurological toxicity was conducted on day 5, 1 month, 6 months, or 1 year after vector delivery. Our data indicate that even at the highest dose tested, real-time quantitative PCR did not detect evidence of biodistribution of HC-Ad genomes to peripheral organs, and behavioral testing did not reveal any vector-mediated abnormalities. Peripheral blood cell counts and serum biochemistry were within normal ranges at all time points, at all doses tested. However, neuropathological assessment revealed evidence of neurotoxicity at the highest dose tested (\(1 \times 10^{10}\) VP of each vector), that is, loss of brain tissue and high levels of inflammation. We did not detect any evidence of neurotoxicity or long-term inflammation at either of the two lower doses tested. HC-Ads are attractive gene delivery platforms for implementing gene therapy strategies for neurological disorders, both chronic neurodegenerative disorders and brain cancer. For successful implementation of these experimental therapies in human patients, it will be necessary to deliver these vectors into the normal brain parenchyma. The data presented herein constitute the first comprehensive study of the biodistribution, behavioral impact, and neuropathological implications after delivery of escalating doses of these vectors into the brain parenchyma, both at acute and chronic time points after vector delivery. Such well-controlled, preclinical safety and toxicity studies are required by the FDA in order to file for a pre-IND/IND and constitute a major milestone in the path toward implementation of the first phase 1 clinical trial for GBM using gutless, HC-Ad vectors. These studies would also support the implementation of HC-Ad vectors to treat other neurological disorders.

**Materials and Methods**

**High-capacity adenoviral vectors**

Details of the molecular characterization, rescue, amplification, and in vitro characterization of the HC-Ad vectors were previously published (Candolfi et al., 2006; Xiong et al., 2006; Muhammad et al., 2010). Briefly, HC-Ad-TK expressed herpes simplex type 1 thymidine kinase (TK) under the control of a constitutive murine cytomegalovirus (mCMV) promoter, and HC-Ad-TetOn-Fli3L expressed human soluble Fms-like tyrosine kinase-3 ligand (Flt3L) under the control of a tightly regulated mCMV-TetOn inducible expression system developed by us (Xiong et al., 2006). HC-Ad vectors used in this study were rescued, amplified, and produced as described previously (Palmer and Ng, 2011). All experiments in the present study were performed in accordance with Good Laboratory Practice (GLP) guidelines. The animal procedures were carried out in accordance with National Institutes of Health (NIH, Bethesda, MD) guidelines for the care and use of laboratory animals and approved by the Cedars-Sinai Medical Center (Los Angeles, CA) Institutional Animal Care and Use Committee. The serum hematological and biochemical analysis was performed at ANTECH Diagnostics (Irvine, CA), which offers clinical pathology services in a regulated environment and is fully compliant with GLP regulations.

**Animals**

Adult male Lewis rats (220–250 g; Harlan, Indianapolis, IN) were used. Rats were kept under controlled conditions of light (12-hr light–dark cycles) and temperature (20–25°C) and fed with standard lab chow and water *ad libitum*.

**Intracranial injections and experiment end points**

Starting 2 days before treatment, rats were fed with doxycycline-mixed chow *ad libitum* (modified LabDiet laboratory rodent diet 5001 with 2000 ppm doxycycline; PMI Nutrition International/Purina Mills, Richmond, IN). Groups of rats were injected unilaterally via the right striatum with any of the three escalating doses of HC-Ad-TK and HC-Ad-TetOn-Fli3L (\(1 \times 10^9\), \(1 \times 10^3\), or \(1 \times 10^{10}\) VP of each vector). The vectors were first mixed together and suspended in a final volume of 3 µl of saline. Using a 10-µl Hamilton syringe fitted with a 26-gauge needle, the vector mixture was administered at the following stereotactic coordinates: 1 mm anterior and 3.2 mm lateral to the bregma and the injection volume of 3 µl was delivered in three locations (1 µl each) at depths of −5.5, −5.0, and −4.5 mm from the dura. Twenty-four hours after treatment, the rats received ganciclovir (GCV, 25 mg/kg, intraperitoneal; Roche Laboratories, Nutley, NJ), twice daily for up to 10 consecutive days. The control group of rats (naive) received 3 µl of saline at the same stereotactic coordinates as the treatment groups.

Groups of rats were evaluated 5 days, 1 month, 6 months, and 1 year after vector delivery to determine biodistribution of HC-Ad vector genomes, neurotoxicity, peripheral blood cell counts and serum biochemistry, and circulating levels of anti-adenovirus neutralizing antibodies and anti-TK antibodies. In addition, the 1-month, 6-month, and 1-year groups were evaluated for HC-Ad vector-induced behavioral...
deficiencies. All animal procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee.

**Serum biochemistry and hematology**

Five days, 1 month, 6 months, or 1 year posttreatment, blood was collected from each animal during euthanasia. The samples were sent to the laboratory of ANTECH Diagnostics (Irvine, CA) for analysis of routine hematological and biochemical parameters. The median, minimal, and maximal values for each parameter are shown in the Results section and supplementary tables (supplementary data are available online at http://www.liebertpub.com/hgtb).

**Circulating neutralizing anti-adenovirus antibodies**

The level of adenovirus-specific neutralizing antibodies was assessed as described previously (Puntel et al., 2010a). Briefly, serum samples were heat-inactivated at 56°C for 30 min and serially diluted 2-fold in minimal essential medium (Invitrogen, Carlsbad, CA) containing 2% fetal bovine serum (FBS). The range of dilutions was 1/2 to 1/4096. Each 50-μl serum dilution was incubated with 1×10⁶ plaque-forming units (PFU) of first-generation adenoviral vector expressing β-galactosidase (Ad-β-Gal) (in a 10-μl volume) for 90 min at 37°C. The 50 μl of sample containing serum and virus was then added to the wells of a 96-well plate containing preseeded (1.5×10⁴) HEK 293 cells per well and were incubated at 37°C for 1 hr. A further 50 μl of medium containing 10% FBS was added to each well, and the cells were incubated at 37°C for 20 hr before fixing with 4% paraformaldehyde in PBS (pH 7.4) and staining with 5-bromo-4-chloro-indolyl-β-d-galactoside (X-Gal; Sigma-Aldrich, St. Louis, MO). The neutralizing antibody titer for each animal is given as the reciprocal of the highest dilution of serum at which 50% of Ad-β-Gal-mediated transduction was inhibited.

**Anti-TK antibody assay**

The titer of anti-TK antibodies in the serum of rats was assessed as described previously (King et al., 2011). Briefly, CNS-1 cells were infected with first-generation adenoviral vector expressing TK (Ad-TK) at a multiplicity of infection of 200 infective units (IU)/cell, or mock infected as a control. After 72 hr, the cells were harvested and freeze-thawed. Cell lysates were added to a 96-well plate (cat. no. 442404; Nunc, Waltham, MA) containing 10% FBS was added to each well, and the cells were incubated at 37°C for 20 hr before fixing with 4% paraformaldehyde in PBS (pH 7.4) and staining with 5-bromo-4-chloro-indolyl-β-d-galactoside (X-Gal; Sigma-Aldrich, St. Louis, MO). The neutralizing antibody titer for each animal is given as the reciprocal of the highest dilution of serum at which 50% of Ad-TK-mediated transduction was inhibited.

**Study of vector genome biodistribution**

Biodistribution of vector genomes was assessed 5 days, 1 month, 6 months, or 1 year posttreatment. This was performed as described previously (Muhammad et al., 2010; Puntel et al., 2010b). Briefly, rats were perfused without any fixative and 25 mg of tissue sample was harvested from the following locations: the brain injection site, contralateral brain hemisphere, cerebellum, brain stem, spleen, liver, testes, small gut, lung, heart, cervical lymph nodes, kidney, and lumbar spinal cord. Total DNA was purified and used for the quantitation of vector genomes by real-time quantitative PCR, using a primer and probe specific for the cosmID sequences contained in the HC-Ad vector backbone as described previously (Puntel et al., 2006). Vector genome data are shown as the ratio of vector genomes per 25 mg of tissue (n=5 per group). Although the limit of detection for the qPCR technique is 100 copies (Puntel et al., 2006), for the qPCR biodistribution studies the limit was determined to be 300 copies. Results from studies in which HC-Ad genome copies were analyzed in the presence of DNA obtained from tissues of naive animals showed that the baseline level of detection was affected, and we thus determined the limit of detection to be 300 copies/25 mg of tissue. We did not encounter any interference in the detection of vector genomes by qPCR in brain tissue; we evaluated this in an experiment in which HC-Ad genomic DNA or pStandard DNA was spiked into brain tissue genomic DNA. In addition, with the purpose of analyzing the biodistribution of Ad vectors as part of an IND application to the FDA, we spiked DNA extracted from all the 13 rat tissues analyzed, either with HC-Ad genomic DNA or the control plasmid, and no interference was detected by qPCR of the specific target DNA for any of the tissues analyzed (our unpublished data).

**Neuropathological analysis**

Neuropathological evaluation was performed 5 days, 1 month, 6 months, or 1 year posttreatment. After perfusion with oxygenated Tyrode’s solution and 4% paraformaldehyde (PFA), brains were postfixed in 4% PFA for an additional 3 days. Sixty-micrometer serial coronal sections were cut in the immediate vicinity of the injection site, and free-floating immunocytochemistry was performed as previously described (Candolfi et al., 2007; King et al., 2008a,b) with markers for oligodendrocytes and myelin sheath (mouse monoclonal anti-MBP [myelin basic protein], cat. no. MAB1580, diluted 1:1000; Chemicon, Temecula, CA), dopaminergic nerve terminals (rabbit polyclonal anti-TH [tyrosine hydroxylase], cat. no. 657012, diluted 1:5000; Calbiochem, La Jolla, CA), CD8+ T cells (mouse anti-CD8, cat. no. MCA48G, diluted 1:1000; Serotec, Raleigh, NC), macrophages and microglia (CD68/ED1, mouse anti-ED1, cat. no. MCA341R, diluted 1:1000; Serotec), macrophages, microglia, and immune cells (mouse anti-MHC-II, cat. no. MCA46G, diluted 1:1000; Serotec), or transgene expression, that is, TK (rabbit anti-TK, custom made, diluted 1:10,000) or Flt3L (rabbit anti-Flt3L, custom made, diluted 1:500). Nissl staining was performed to assess gross histopathological features of the brains. The stained sections were photographed with a Carl Zeiss optical Axiosplan microscope using Axiovision release 4.6 and Mosaix software (Carl Zeiss, Chester, VA).
Behavioral analysis

The neurobehavioral impact as a consequence of treatment using escalating doses of HC-Ad was assessed 1 month, 6 months, and 1 year posttreatment. Testing of amphetamine-induced rotational behavior, abnormalities in limb use asymmetry, and spontaneous motor and rearing behavior was done as described previously in detail (King et al., 2008b). Briefly, amphetamine-induced rotational behavior was measured with a RotoMax apparatus and software (AccuScan Instruments, Columbus, OH) for 90 min after subcutaneous injection of d-amilphetamine sulfate (1.5 mg/kg; Sigma-Aldrich). To measure forelimb use asymmetry, contacts made by each forepaw with the wall of a 20.3-cm-wide clear cylinder were scored from videotape over a 10-min period in slow motion by two independent, experimentally blinded observers. Forelimb contact with the walls of the cylinder was scored by measuring only initial contact with the cylinder walls. Baseline spontaneous locomotor and rearing activity was recorded for 30 min in a 40.6 × 40.6 × 38.1 cm closed box, using photobeam breaks and optical sensors. Spontaneous locomotor and rearing activity was then monitored for 120 min after subcutaneous injection of d-amilphetamine sulfate (1.5 mg/kg; Sigma-Aldrich). Data were scored as the total number of beam breaks summed over the observation period.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA). Data were compared by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. p values less than 0.05 were considered as significant.

Results

Dose escalation of HC-Ad-TetOn-Flt3L plus HC-Ad-TK delivered into naive rat brain followed by neuropathological analysis

To examine the effects of HC-Ad-TetOn-Flt3L and HC-Ad-TK on normal brain architecture and inflammation, we performed a comprehensive neuropathological analysis 5 days, 1 month, 6 months, and 1 year after intrastratial delivery of HC-Ads. Naive adult Lewis rats received a combination of either 1 × 10^8, 1 × 10^9, or 1 × 10^10 VP of each HC-Ad vector or saline (Fig. 1a). GCV was administered twice daily for up to 10 days. The rats were fed doxycycline-containing chow for up to 4 weeks because this schedule was found to be optimal for therapeutic efficacy (Muhammad et al., 2010). Neuropathological assessment revealed evidence of neurotoxicity at the highest dose tested (1 × 10^10 VP of each HC-Ad; total dose, 2 × 10^10 VP), that is, loss of brain tissue and high levels of inflammation. We did not encounter any evidence of brain tissue damage at either of the lower doses tested. Nissl staining was more prominent 5 days after delivery of the highest dose of HC-Ad when compared with saline-treated controls and rats receiving either of the lower vector doses (1 × 10^8 and 1 × 10^9 VP of each vector) (Fig. 1b). Myelin basic protein and tyrosine hydroxylase immunoreactivity also indicated the presence of tissue damage with the high total dose of 2 × 10^10 VP. There was profuse infiltration of CD8+ T cells, macrophages, and MHC-II+ cells in the injected brain hemisphere in all groups, including saline-treated controls, at the acute 5-day time point. However, the observed immune cell infiltration was much more pronounced with the highest dose (1 × 10^10 VP of each HC-Ad) (Fig. 1b). In addition, there was mild CD68/ED1 and MHC-II immunoreactivity seen in the contralateral brain hemisphere of saline-treated and all vector-treated groups at the early time point, that is, 5 days. The immune cell infiltration seen in all groups declined gradually over time, that is, at 1 month, 6 months, and 1 year after treatment (Fig. 1c; and see Supplementary Fig. S1); no CD8+ T cells were observed 1 year after treatment with either of the lower doses (1 × 10^8 and 1 × 10^9 VP of each vector) or saline (Fig. 1c). Regarding expression of the transgene proteins in the brain, immunocytochemical analysis revealed an abundance of cells expressing TK and Flt3L on day 5 after treatment (Fig. 1b); the presence of transgene-expressing cells was also evident at 1 and 6 months (Supplementary Fig. S1) and even at 1 year (Fig. 1c) after HC-Ad delivery. High-power images are provided for Nissl staining at all doses and time points tested (Fig. 2). These neuropathological data indicate that 1 × 10^9 VP of each HC-Ad is the maximal tolerated dose (MTD) that can be safely administered into the brain of naive rats.

HC-Ad-TetOn-Flt3L and HC-Ad-TK (plus GCV)-treated animals do not exhibit neurobehavioral deficits

Because the gene therapy vectors will be delivered into the surrounding brain parenchyma after tumor resection in patients with GBM, it is important to assess the occurrence of any acute and/or chronic neurological deficits elicited by HC-Ads. To this end, adult naive Lewis rats treated with escalating doses of HC-Ad-TetOn-Flt3L and HC-Ad-TK were subjected to a panel of neurobehavioral tests 1 month, 6 months, and 1 year after treatment. Analysis of amphetamine-induced total locomotor activity (Fig. 3a; and see Supplementary Figs. S2a and S3a), total rearing activity (Fig. 3b; and see Supplementary Figs. S2b and S3b), and rotational behavior (Fig. 3d; and Supplementary Fig. S3d) did not reveal any abnormalities in the HC-Ad-treated rats when compared with saline-treated age-matched naive controls (one-way ANOVA followed by Tukey’s multiple comparison test). The right limb use asymmetry test (Fig. 3c; and see Supplementary Fig. S3c) also did not reveal any abnormalities except for the groups treated with the highest dose (1 × 10^10 VP of each HC-Ad) when compared with rats treated with the lowest dose (1 × 10^8 VP of each HC-Ad) at 1 month (one-way ANOVA followed by Tukey’s multiple comparison test; p < 0.05) (Supplementary Fig. S2c).

Biodistribution of HC-Ad vector genomes is restricted to the injected brain hemisphere at all time points and HC-Ad vector doses tested

The biodistribution of HC-Ad vector genomes was assessed in the brain, spinal cord, and peripheral organs 5 days, 1 month, 6 months, and 1 year after HC-Ad vector delivery, using quantitative PCR analysis. At all doses and time points tested, HC-Ad genomes were restricted to the injection site in the ipsilateral hemisphere (Fig. 4; and see Supplementary Fig. S4). HC-Ad vector genomes were below detectable limits in all peripheral organs, including other regions of the brain and spinal cord, even at the highest dose.
FIG. 1. Experimental design and neuropathological analysis after treatment with HC-Ads. (A) Diagram of experimental design used. Adult naive Lewis rats were injected stereotactically in the right striatum with either saline or escalating doses of high-capacity adenoviral vectors, HC-Ad-TK plus HC-Ad-TetOn-Flt3L ($1 \cdot 10^8$, $1 \cdot 10^9$, or $1 \cdot 10^{10}$ VP of each vector); after 24 hr, they received ganciclovir (GCV; 25 mg/kg, intraperitoneal), twice daily for up to 10 days. All rats were fed doxycycline mixed rodent chow (Dox chow) ad libitum for up to 4 weeks starting 2 days before treatment. Groups of rats were evaluated at 5 days (short-term), 30 days (medium-term), 180 days or 365 days (long-term) for biodistribution of vector genomes, neuropathology, peripheral blood cell counts and serum biochemistry, circulating levels of anti-adenovirus neutralizing antibodies, and anti-TK antibodies. In addition, the 30-day, 180-day, and 365-day posttreatment groups underwent neurobehavioral testing. The rats were killed at experiment end points and organs were harvested for processing. Neuropathological analysis was performed with the brains of rats at 5 days (B) and at 1, 6, and 12 months (C). Neuropathology results at 1 and 6 months are shown in Supplementary Fig. S1. The brains were processed for Nissl staining to show gross morphology, and immunocytochemistry with primary antibodies against myelin basic protein (MBP) to label oligodendrocytes and myelin sheaths, tyrosine hydroxylase (TH) to label striatal dopaminergic fibers, CD8 to detect CD8$^+$ T cells, CD68/ED1 for labeling macrophages and activated microglia, MHC-II to label MHC-II$^+$ macrophages, microglia, and immune cells, thymidine kinase (TK) or Fms-like tyrosine kinase-3 ligand (Flt3L) to detect expression of therapeutic transgenes in the striatum. Scale bar: 1000 μm for all but TK and Flt3L: 250 μm.
tested at all time points, thereby highlighting the high safety profile of this HC-Ad-mediated, combined gene therapy approach.

**Circulating neutralizing anti-adenovirus antibodies and anti-thymidine kinase antibodies in the serum of HC-Ad-treated animals**

Blood samples were collected from each animal during euthanasia to measure anti-adenovirus neutralizing antibodies and anti-thymidine kinase antibodies. Serum levels of anti-Ad neutralizing antibodies were indistinguishable from background levels at all time points with total HC-Ad doses of $2 \times 10^8$ and $2 \times 10^9$ VP. Animals injected with the highest dose of HC-Ads ($2 \times 10^{10}$ VP) exhibited detectable, albeit low titers of circulating anti-Ad neutralizing antibodies, consistent with the generation of antibodies against adenoviral proteins, possibly due to some degree of leakage of the HC-Ads into the ventricular space (Fig. 5). The highest titers were exhibited 1 month after vector delivery, ~1:64, decreasing to titers below 1:20 at 6 months and 1 year after HC-Ad delivery (Fig. 5). Antibodies against thymidine kinase were also elevated at 1 year in the serum of animals injected with the highest dose of HC-Ads (Fig. 6) (one-way ANOVA followed by Tukey’s multiple comparison test; $p<0.05$).

**Analysis of blood biochemistry and clinical laboratory parameters**

To detect any possible systemic side effects after delivery of escalating doses of therapeutic HC-Ads in the brain of naive Lewis rats, serum biochemistry and blood cell counts were carried out on blood samples harvested 5 days, 1 month, 6 months, and 1 year posttreatment. Serum biochemical parameters indicated normal liver and renal functions; the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, urea, and creatinine were found to be within the normal range as seen in age-matched naive Lewis rats, at all time points and vector doses tested (Table 1; and see Supplementary Tables S1–S3). Red and white blood cell counts in the treated animals were also within normal ranges, indicating that Flt3L expression in the naive rat brain does not substantially alter the levels of circulating immune cells.
FIG. 3. Behavioral assessment of rats 1 year after treatment with high-capacity adenoviral vectors, HC-Ad-TK plus HC-Ad-TetOn-Flt3L. Behavioral assessment was performed before and after amphetamine treatment in the animals, at 1 month (Supplementary Fig. S2), 6 months (Supplementary Fig. S3), and 1 year (Fig. 2) after intracranial administration of escalating doses of HC-Ad-TK plus HC-Ad-TetOn-Flt3L. Saline-treated, age-matched rats were used as controls. (a) Total locomotor activity; (b) total rearing activity; (c) right limb use asymmetry; (d) asymmetry in rotational behavior.

Discussion

There is accumulating evidence that supports the use of immunotherapies in combination with standard anti-GBM treatment modalities, that is, surgery, radiotherapy, and chemotherapy (Sul and Fine, 2010; Vauléon et al., 2010; Castro et al., 2011; Heimberger and Sampson, 2011; Szabo and Carpentier, 2011; Weller, 2011). We have developed an effective immune-gene therapy strategy for treating GBM that comprises the combined use of conditional cytotoxicity (HSV1-TK plus systemic delivery of GCV) (Dewey et al., 1999) and immune stimulation elicited by expressing human Flt3L (hFlt3L) within the tumor microenvironment (Ali et al., 2004; Curtin et al., 2006). hFlt3L is effective in rodents; we have shown its efficacy to treat intracranial tumors and elicit anti-tumor-specific immune responses in several intracranial GBM preclinical models in both rats and mice (Ali et al., 2004, 2005; King et al., 2008a,b; Candolfi et al., 2009; Curtin et al., 2009; Ghulam Muhammad et al., 2009; Mineharu et al., 2011). Further, we have also shown that hFlt3L is capable of recruiting antigen-presenting cells into the brain microenvironment after adenovirus-mediated delivery into the striatum of rats (Curtin et al., 2006). Our treatment strategy involves availability of tumor antigens and innate immune adjuvants such as high-mobility group B1 protein (HMGB1) from dead/dying GBM cells initiated by TK (plus GCV) and uptake of the tumor antigens by antigen-presenting cells (dendritic cells, DCs), which are recruited into the tumor microenvironment by Flt3L (Curtin et al., 2006, 2009). This combined gene therapy approach also generates immunoLOGical memory that protects against recurrent GBM. It is also effective in models of multifocal, and peripheral GBMs (King et al., 2008a,b, 2011; Ghulam Muhammad et al., 2009).

It has been established that there is a high prevalence of systemic immunity against Ads in the human population (Chirmule et al., 1999; Nwanegbo et al., 2004; Sumida et al., 2005), and this could potentially interfere with and compromise the efficacy of gene therapies mediated by therapeutic Ad-based vectors (Sun et al., 2011). To address this problem, we developed HC-Ads as delivery platforms for the therapeutic transgenes TK and Flt3L (Candolfi et al., 2006; Xiong et al., 2006; King et al., 2008c; Muhammad et al., 2010). HC-Ads have numerous advantages, including their large cloning capacity, negligible toxicity, and long-term transgene expression profile (Parks et al., 1996; Schiedner et al., 1998; Morrall et al., 1999; Thomas et al., 2001a,b; Palmer and Ng, 2005; Schillinger et al., 2005; Toietta et al., 2005; Barcia et al., 2007). The HC-Ads used in our study were grown with a helper virus that provides all essential adenoviral functions for replication in trans. The packaging sequence of the helper virus is flanked by loxP recombination sites, in 293-Cre cells. The helper viral genome undergoes recombination that deletes the packaging sequence, and thus the helper virus genome becomes less efficiently packaged than the HC-Ad genome (Parks et al., 1996; Palmer and Ng, 2003, 2004). With this production method, titers of contaminating helper virus are consistently ~0.05% of HC-Ad genomes in the final preparations (Ng et al., 2001; Palmer and Ng, 2003, 2004; Puntel et al., 2006). Given that in our study the highest dose injected into the brain was 1×1010 VP, the helper virus contamination would be less than 5×106 VP. Furthermore, we have previously shown negligible levels of inflammation triggered by the injection of HC-Ad into the naive brain, even in the presence of a systemic anti-Ad immune response (Thomas et al., 2000, 2001b; Xiong et al., 2006; Barcia et al., 2007; King et al., 2008c). This study involves the use of two HC-Ad vectors: HC-Ad-TK, which expresses TK.
constitutively (Candolfi et al., 2006; King et al., 2008c; Muhammad et al., 2010), and HC-Ad-TetOn-Flt3L, which expresses Flt3L under the tight control of a regulatable mCMV-TetOn expression system (Candolfi et al., 2006; Muhammad et al., 2010). We have considered putting both treatment cassettes into the same vector, and the efficacy data using the bicistronic HC-Ad encoding both therapeutic transgenes have been published previously (Puntel et al., 2010b). Both approaches have advantages and disadvantages. The rationale for testing the two-separate-vector approach is, first, that we can dose each vector/therapeutic transgene independently. This will be an advantage in the setting of clinical trials as the toxicity of each vector can be tested on its own. For the design of the phase 1 trial we will propose a dose-finding safety trial in which the dose of each vector will be varied individually. Also, because the phase 1 trial using the combined TK/Flt3L strategy has been approved by the FDA using two independent first-generation Ad vectors (14574 IND approved 4/7/11), it was deemed prudent to keep the clinical trial design as planned and change only perceived safety issues, that is, the vector backbone, and the addition of a regulatable transcriptional switch to turn the expression of Flt3L "on" and "off" according to clinical need. Second, there may be an advantage to expressing the therapeutic transgenes in different cells, because the dividing cells that are expressing TK will be killed in the presence of GCV, and thus, if Flt3L is expressed in that same cell, this would diminish the expression of the immune stimulatory arm of the therapy. Nevertheless, as a single high-capacity Ad vector encoding both transgenes has been produced by us, we will be able to test it in clinical trials in the future.

We used the CNS-1 GBM rat model to test the safety and efficacy of the engineered HC-Ads encoding Flt3L and TK delivered into the tumor mass (Muhammad et al., 2010). This HC-Ad-mediated anti-GBM gene therapy was also tested in GBM rat models that were immunized by intradermal injection of an empty Ad vector (i.e., does not express any transgene). These immunized rats displayed high levels of circulating neutralizing anti-Ad antibodies, thereby mimicking the likely scenario to be encountered during human clinical trials. We found that the GBM was eradicated in ~70% of rats within 30 days of treatment and that the therapeutic strategy was effective even in the presence of a...
FIG. 5. Anti-adenovirus neutralizing antibodies in the serum of HC-Ad-treated rats. The prevalence of anti-adenovirus neutralizing antibody in the serum of rats treated with intracranial injections of escalating doses of HC-Ad-TK plus HC-Ad-TetOn-Flt3L or saline is shown. Ganciclovir (25 mg/kg, intraperitoneal) was administered twice daily for up to 10 days and doxycycline (Dox) chow was administered ad libitum for up to 4 weeks. The rats were killed 5 days (a), 1 month (b), 6 months (c), and 1 year (d) after treatment and sera were collected. Low levels of neutralizing antibody were detected at the highest dose ($1 \times 10^{10}$ VP) after 1 month of HC-Ad delivery. *$p < 0.05$ versus saline or either of the lower vector doses (one-way ANOVA followed by Tukey’s multiple comparison test).

FIG. 6. Circulating anti-HSV1-TK antibodies in the serum of HC-Ad-treated rats. Escalating doses of HC-Ad-TK plus HC-Ad-TetOn-Flt3L, or saline, were stereotactically injected into the striatum of Lewis rats. Ganciclovir (25 mg/kg, intraperitoneal) was administered twice daily for up to 10 days and doxycycline (Dox) chow was administered ad libitum for up to 4 weeks. The rats were killed 5 days (a), 1 month (b), 6 months (c), and 1 year (d) after treatment and sera were collected. Serum from each animal was evaluated for the presence of circulating antibodies specific for thymidine kinase (TK). Circulating anti-TK antibodies were detected at the highest dose ($1 \times 10^{10}$ VP) 1 year after HC-Ad delivery into the naive rat brain. The titer of anti-TK antibodies is represented as the percent change in signal intensity from sera incubated with cell lysates from Ad-TK-infected cells when compared with mock-infected cells. Samples were considered positive when the percent change was $\geq 25$%; this threshold is indicated by the dashed line. *$p < 0.05$ versus saline or either of the lower vector doses (one-way ANOVA followed by Tukey’s multiple comparison test).
Table 1. Biochemical and Hematological Parameters 5 Days Posttreatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Naive</th>
<th>Saline</th>
<th>HC-TK + HC-Flt3L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median Range</td>
<td>Median Range</td>
<td>Median Range</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HC-TK + HC-Flt3L</td>
<td></td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>5.7 (5.6–6.1)</td>
<td>5.8 (5.7–7.1)</td>
<td>5.9 (5.6–6.2)</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>2.9 (2.8–3)</td>
<td>2.9 (2.5–3)</td>
<td>2.8 (2.4–3.2)</td>
</tr>
<tr>
<td>Globulin (g/dl)</td>
<td>2.8 (2.7–3.1)</td>
<td>3 (2.8–4.2)</td>
<td>3 (2.6–3.4)</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>87 (63–222)</td>
<td>110 (67–593)</td>
<td>95 (70–203)</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>51.5 (46–89)</td>
<td>41 (30–161)</td>
<td>37 (28–68)</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.1 (0.1–0.1)</td>
<td>0.2 (0.1–0.6)</td>
<td>0.2 (0.1–0.3)</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>19.5 (18–21)</td>
<td>24 (16–85)</td>
<td>23 (14–37)</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.3 (0.2–0.3)</td>
<td>0.5 (0.4–1.5)</td>
<td>0.4 (0.3–0.7)</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>8.1 (7.2–11.2)</td>
<td>13.9 (9.6–22.1)</td>
<td>11.3 (8.2–18.6)</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>86.5 (47–105)</td>
<td>162 (57–310)</td>
<td>189 (109–473)</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>10.4 (9.9–10.9)</td>
<td>10.6 (8.9–11.5)</td>
<td>10.4 (9.6–40)</td>
</tr>
<tr>
<td>Sodium (mmol/dl)</td>
<td>142.5 (140–144)</td>
<td>138 (134–141)</td>
<td>140 (136–143)</td>
</tr>
<tr>
<td>Potassium (mmol/dl)</td>
<td>6.3 (5.4–7.2)</td>
<td>10.6 (8.1–13.4)</td>
<td>8.3 (5.6–13.9)</td>
</tr>
<tr>
<td>Chloride (mmol/dl)</td>
<td>98 (97–99)</td>
<td>98 (92–101)</td>
<td>100 (96–105)</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>70–118</td>
<td>79 (73–93)</td>
<td>77 (67–93)</td>
</tr>
<tr>
<td>CPK (IU/L)</td>
<td>675.5 (170–7,517)</td>
<td>493 (193–23,079)</td>
<td>490 (194–5,747)</td>
</tr>
<tr>
<td>WBC count (× 10^9 /l)</td>
<td>8.8 (7.3–10)</td>
<td>5.8 (4.1–7.6)</td>
<td>6.6 (4.9–8.7)</td>
</tr>
<tr>
<td>RBC count (× 10^6 /l)</td>
<td>8.2 (7.9–8.6)</td>
<td>9 (8.2–9.5)</td>
<td>9.2 (8.6–9.9)</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.7 (13.3–14.6)</td>
<td>15 (14–15.8)</td>
<td>15.4 (14.2–16.3)</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>51 (49–52)</td>
<td>55 (52–62)</td>
<td>57 (56–60)</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>16.8 (16.5–17.5)</td>
<td>16.6 (15.9–17.1)</td>
<td>16.7 (15.8–17.1)</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>13 (10–15)</td>
<td>18 (9–32)</td>
<td>13 (9–22)</td>
</tr>
<tr>
<td>Absolute Neutrophils (/µl)</td>
<td>1074 (870–1,500)</td>
<td>928 (369–2,432)</td>
<td>858 (490–1,496)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>85 (83–87)</td>
<td>79 (64–89)</td>
<td>84 (75–90)</td>
</tr>
<tr>
<td>Absolute lymphocytes (/µl)</td>
<td>7,611.5 (6,059–8,300)</td>
<td>4,814 (3,649–5,808)</td>
<td>5,544 (4,263–7,396)</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>1 (1–2)</td>
<td>1 (1–3)</td>
<td>2 (1–4)</td>
</tr>
<tr>
<td>Absolute monocytes (/µl)</td>
<td>95.0 (87–174)</td>
<td>58 (41–228)</td>
<td>132 (55–261)</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1 (0–1)</td>
<td>1 (0–2)</td>
<td>1 (0–1)</td>
</tr>
<tr>
<td>Absolute eosinophils (/µl)</td>
<td>88.0 (100–100)</td>
<td>52 (0–94)</td>
<td>60 (0–86)</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0 (0–0)</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>Absolute basophils (/µl)</td>
<td>0 (0–0)</td>
<td>0 (0–66)</td>
<td>0 (0–73)</td>
</tr>
<tr>
<td>Platelet (/× 10^9 /µl)</td>
<td>488.5 (337–666)</td>
<td>793 (746–968)</td>
<td>1,014 (917–1,167)</td>
</tr>
</tbody>
</table>

Note: Red blood cell morphology appeared normal in all groups.

AST, aspartate aminotransferase; ALT, alanine transaminase; BUN, blood urea nitrogen; CPK, creatine phosphokinase; WBC, white blood cell; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.
systemic antiadenoviral immune response. Further, long-term immunological memory protected the cured rats against tumor rechallenge, even 1 year after treatment (Muhammad et al., 2010), highlighting the therapeutic efficacy of this approach in countering recurrences of GBM.

Although our combination therapy with HC-Ad-TK and HC-Ad-TetOn-Flt3L was found to be highly effective and safe when injected to treat intracranial GBM, the tumor microenvironment is well known to possess unique structural features that are significantly different from those of the surrounding brain tissue (Lorusso and Ruegg, 2008; Pietras and Ostman, 2010; Charles et al., 2011). Hence, it was critical to conduct the present study to perform a thorough assessment of the neuropathology, biodistribution and transgene expression, behavioral impact, as well as any putative systemic and toxicity of HC-Ads when delivered within the normal adult rat brain parenchyma. Accordingly, escalating doses of HC-Ads (1 × 10^6, 1 × 10^7, or 1 × 10^8 VP of each HC-Ad) were injected into the right striatum and groups of rats were killed on day 5, 1 month, 6 months, or 1 year post-treatment and tissue samples were harvested for analysis. Rats belonging to the 1-month, 6-month, and 1-year post-treatment groups were also subjected to a panel of neurobehavioral tests.

Histopathological analysis of the brain sections did not reveal any evidence of neurotoxicity or long-term inflammation at the lower doses tested (total HC-Ad dose, 2 × 10^6 or 2 × 10^7 VP). At the highest dose tested, that is, a total HC-Ad dose of 2 × 10^10 VP, we observed loss of brain tissue and high levels of inflammation, suggesting that this dose is unsafe to the naive rat brain. Infiltrating immune cells were localized in the vicinity of the injection site in the ipsilateral hemisphere, comprising cells immunopositive for CD8, CD68/ED1, and MHC-II. Infiltrating immune cells decreased at 6 months and 1 year after HC-Ad vector injection; this may be attributed in part to the unique regulatable features engineered into the vector that expresses the transgene Flt3L, that is, its expression is triggered only in the presence of doxycycline (Dox). The most likely explanation for the inflammatory and/or immune mechanisms observed at the highest dose tested is that the toxicity seen has a similar basis as that described for first-generation adenoviral vectors (Thomas et al., 2001a). The highest total dose of HC-Ad tested (2 × 10^10 VP) is cytotoxic to the brain at the earliest time point, that is, 5 days; the progressive elimination of affected brain tissue can be detected as a slow shrinkage of the striatum and enlargement of the ventricles, observed after 1 and 6 months, and is fully established after 12 months. It is unlikely that a T cell response would have been able to play any significant role in this cytotoxicity. Given that we used a gutted adenoviral vector, the turnover of input viral capsids and the incapacity of infected cells to continue to produce capsid proteins, would effectively make any infected cells invisible to anti-viral vector T cells (Barcia et al., 2007). We have no evidence that immune responses against the transgenes were ever generated in our experimental systems. We decided to test the toxicity of the combined HC-Ads because in human patients undergoing phase 1 clinical trials for GBM, the vectors will be delivered in combination. The toxicities seen at the highest doses are likely elicited by the high levels of viral protein capsids and not by the therapeutic transgenes (Gerdes et al., 2000; Thomas et al., 2001a; Hurtado- Lorenzo et al., 2003; Lowenstein and Castro, 2003, 2004; Barcia et al., 2006). Also, we evaluated putative toxicities of the therapeutic HC-Ad vectors only in the presence of ganciclovir or Dox, because in human patients the vectors will be administered in conjunction with these drugs delivered systemically. However, in previous studies in rodents it was shown that ganciclovir does increase the toxicity somewhat (Cowsill et al., 2000); nevertheless, the main factor in adenoviral vector toxicity remains the vector capsid itself, with ganciclovir playing a minor role, mainly due to the killing of any dividing cells in the CNS.

It is not known whether infection with HSV will cause an immune response against the HSV1-TK, which could cross-react with TK being expressed in the brain from the gutted adenoviral vector genomes. However, during HSV2 infection the immune response is directed mainly against envelope glycoproteins rather than late proteins, such as HSV1-TK. We have previously shown that in animals preimmunized with first-generation adenoviral vectors, high-capacity adenoviral vector expression lasts for at least 12 months in the absence of neurotoxicity in the CNS (Barcia et al., 2007). We have also previously shown that immunization with first-generation adenoviral vectors of animals injected via the brain with high-capacity adenoviral vectors 2 months earlier, fails to cause brain inflammation. We have not looked for expression of helper virus products because the amount of helper virus injected is less than 5 × 10^7; it would be technically difficult to detect expression of any protein expressed from the helper virus genome. We have previously also shown that an Ad dose of 1 × 10^6 IU delivered into the brain does elicit low levels of transgene expression and no local inflammation, and that more than 1 × 10^7 IU must be injected into the subcutaneous tissue to cause a systemic immune response against adenovirus (Barcia et al., 2007).

The biodistribution data revealed that HC-Ad vector genomes were confined to the brain injection site and did not leak to other areas of the brain, spinal cord, or peripheral organs. Interestingly, sustained levels of HC-Ad vector genomes were seen to persist in the brain injection site for up to 1 year after treatment, suggesting that the cytotoxic effects of TK or the immune-stimulatory effects of Flt3L could be “reactivated” with the readministration of GCV or Dox, respectively, if required.

Peripheral blood cell counts were found to be within the normal range and serum biochemical profiles indicated normal liver and renal functions in rats at all time points and doses tested. In the groups treated with either escalating HC-Ad doses or saline, some rats showed higher platelet counts in comparison with age-matched naive untreated rats. However, the platelet counts were similar in both the HC-Ad- and saline-treated controls. Some blood glucose levels were also seen to be elevated in the treatment groups but appeared to be similar in both HC-Ad-treated and saline-treated controls. Analysis of amphetamine-induced total locomotor activity and total rearing activity also did not reveal any behavioral abnormalities when compared with saline-treated age-matched naive controls. We detected low levels of neutralizing antibodies 1 month after HC-Ad delivery, in the serum of animals injected with the highest dose of HC-Ads; antibodies against thymidine kinase were also slightly elevated 1 year postinjection in the serum of animals injected with the highest dose of HC-Ads. This could be because,
although the immune-privileged status of brain can hamper immune responses against vectors delivered into the brain parenchyma, leakage into the intraventricular space allows systemic exposure because the cerebrospinal fluid drains to the venous circulation, thereby raising the chance of initiating an antivector immune response. In previous studies, we have examined preimmunized rats for signs of neurotoxicity. This was done in the context of achieving long-term expression (Barcia et al., 2007), or in the context of the treatment of brain tumors (Muhammad et al., 2010). In the naive brain, expression from high-capacity Ad vectors stretched to 12 months in the absence of any untoward effects. Equally, gutted adenoviruses were able to eliminate tumors from animals preimmunized against adenoviral vectors in the absence of overt neurotoxicity (King et al., 2008c). Non-immunized and preimmunized tumor-bearing rats were treated with HC-Ad-TK and first-generation Ad-TK and the results show that HC-Ad-TK induced tumor regression and long-term survival in both nonimmunized and preimmunized rats. However, Ad-TK treatment failed in the preimmunized rats (King et al., 2008c). HC-Ad-TK was efficacious when used in combination with tHC-Ad-TetOn-Flt3L to treat preimmunized rats with large brain tumors (Muhammad et al., 2010). HC-Ad-TetOn-Flt3L and HC-Ad-TK therapy improved survival of tumor-bearing animals (naive and preimmunized) having a preexisting anti-Ad immune response (Muhammad et al., 2010).

The analyses performed suggest that the overall safety profile of the gutted adenoviral vectors, HC-Ads encoding TK, does match the earlier studies using first-generation Ad-TK vectors. This is to be expected, as most of the toxicity is due to the viral capsids, and these are identical for both vectors. Also, in another study, it has been shown that both neurotoxicity and systemic toxicity with Ad-TK is similar to those with HC-Ad-TK (Candolfi et al., 2009). Furthermore, the gutted adenoviral vectors have an advantage when used in animals that have been preimmunized against adenovirus. We have previously shown that treatment of a syngeneic intracranial glioma model with HC-Ad-TK induced tumor regression and long-term survival in both nonimmunized and preimmunized tumor-bearing rats, whereas treatment with Ad-TK failed in preimmunized tumor-bearing rats (King et al., 2008c). HC-Ad-TK was efficacious when used in combination with HC-Ad-TetOn-Flt3L to treat preimmunized rats with large brain tumors (Muhammad et al., 2010). HC-Ad-TetOn-Flt3L and HC-Ad-TK therapy improved survival of tumor-bearing rats exhibiting a preexisting anti-Ad immune response. Ad-TK/Ad-Flt3L, however, was not efficacious in preimmunized rats (Muhammad et al., 2010).

The present study, in conjunction with previously published work from our laboratory and others, highlights that GBM and other malignant brain tumors are attractive “target” diseases for implementing gene therapeutic strategies. In this respect, delivery of viral vectors in the brain is well established, both in animal models and also in human patients enrolled in clinical trials (Immonen et al., 2004; Markert et al., 2009). Also, the therapeutic efficacy of HC-Ad vectors expressing transgenes targeted to treat disease conditions such as brain cancer, colorectal cancer, hyperbilirubinemia, monogenic hypoalphalipoproteinemia, hemophilia, diabetic retinopathy, glycogen storage disease, hypertension, and sensory neuronopathies has been assessed in preclinical models showing high efficacy (Schillinger et al., 2005; Toietta et al., 2005; Kiang et al., 2006; McCormack et al., 2006; Lamartina et al., 2007; Oka et al., 2007; Terashima et al., 2009; Muhammad et al., 2010; Punet et al., 2010a; Gonzalez-Aparicio et al., 2011). Thus, this comprehensive, preclinical safety and toxicity study will facilitate the implementation of phase 1 clinical trials for GBM using high-capacity adenoviral vectors; it will also lay the foundations for the treatment of other devastating neurological diseases using HC-Ad-mediated gene therapy strategies.

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Author Disclosure Statement

The authors declare they have no conflict of interest.

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