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High-Capacity adenoviral vector-mediated anti-glioma gene therapy in the presence of systemic anti-adenovirus immunity.

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Abstract

Gene therapy is proposed as a novel therapeutic strategy for glioblastoma (GBM), a devastating brain cancer. In the clinic, anti-vector immune responses pose formidable challenges. Herein we demonstrate that high-capacity adenovirus vectors (HC-Ad) encoding the conditional cytotoxic gene, herpes simplex virus type 1-thimidine kinase (TK) induce tumor regression and long term survival in an intracranial glioma model, even in the presence of systemic anti-adenovirus immunity as could be encountered in patients. First generation Ad-TK fails to elicit tumor regression in this model. These results pave the way for implementing HC-Ad-TK mediated gene therapy as a powerful adjuvant for treating GBM.

Glioblastoma multiforme (GBM) is a malignant primary brain tumor associated with a <2% survival five years post-diagnosis. Advances in neurosurgery, chemotherapy, and radiotherapy have not impacted the dire clinical statistics; therefore, novel therapies are urgently needed. Adenovirus-mediated delivery of the conditional cytotoxic gene, herpes simplex virus type 1-thymidine kinase (HSV1-TK) has been proposed as an adjuvant gene therapy approach for GBM (7, 8). HSV1-TK is non-toxic to humans; only actively dividing cells expressing HSV1-TK will convert intravenously administered gancyclovir (GCV) into cytotoxic nucleotides that will kill proliferating GBM cells. This approach has been tested in humans using first generation adenoviral vectors (Ads) with optimal
transduction efficiency achieved when the Ads were delivered at multiple injection sites (16). Results from Phase II trials are encouraging with a 65% increase in median survival of Ad-TK treated patients when compared to control groups, although median life expectancy was only increased to 62.4 weeks (8). Results from a large multicentric Phase III trial are awaited (16). The significant, yet limited success elicited after delivering Ad-TK to the tumor mass in situ, or tumor bed following resection, (8) is likely due to the presence of pre-existing systemic immune responses against adenoviruses in many human patients (6). Anti-adenoviral immune responses would hamper therapeutic transgene expression from first generation Ad vectors, resulting in diminished clinical efficacy of the treatment, when compared to the success attained in preclinical models (1, 7, 8). Along these lines, it has been recently shown that adeno-associated virus (AAV)-serotype 2 mediated hepatic gene transfer results in transgene product expression which is stable in pre-clinical animal models, but is short lived, declining 4-6 weeks post-AAV2 delivery in human patients (13). This decline was caused by elimination of transduced hepatocytes by AAV vectors, mediated by AAV-2 capsid-specific CD8⁺ T cells (13).

The new generation ‘gutless’, high-capacity adenovirus (HC-Ad) vectors, have a significantly favorable immunological profile (2, 4, 19). Even in the presence of a pre-existing systemic anti-adenoviral immune response that eliminates transgene expression from first generation Ads, transgene expression from HC-Ad vectors remains stable for up to one year (2, 12, 14, 18-20). In this report, using a
syngeneic model of intracranial GBM, we demonstrate that intratumoral delivery
of HC-Ad-TK in combination with peripheral administration of GCV elicited GBM
regression and long term survival, even in the presence of a systemic pre-
existing immune response against Ads as is likely to be encountered in the
clinic(6). Intratumoral delivery of Ad-TK completely fails to improve long term
survival in tumor bearing animals which had been pre-immunized against Ads.
Further, therapeutic efficacy in the presence of systemic anti-Ad immunity
ensued without overt neuropathological side effects, following intratumoral
administration of HC-Ad-TK. Our data suggest that this gene therapeutic
approach could be a powerful adjuvant for the treatment of GBM, even in
patients who would have been pre-exposed to adenovirus.

All experimental manipulations in Lewis rats were approved by the Institutional
Animal and Care Committee (IACUC) of Cedars Sinai Medical Center/UCLA. To
assess the anti-GBM therapeutic efficacy of HC-Ad-TK and Ad-TK in the
presence of systemic anti-Ad immunity, we utilized a first generation E1/E3
deleted vector (Ad-TK) and a helper dependent, high capacity adenoviral vector
(HC-Ad-TK), both constitutively expressing HSV1-TK under the control of the
powerful murine CMV (mCMV) promoter (1, 5, 15, 17). The characteristics of
Ad-TK were: total viral particles: $1.15 \times 10^{13}$ vp/ml; vector genomes: $1.19 \times 10^{13}$
vg/ml and infectious units: $1.46 \times 10^{12}$ iu/ml. For HC-Ad-TK, total viral particles:
$6.15 \times 10^{12}$ vp/ml; vector genomes: $4.90 \times 10^{12}$ vg/ml and helper virus
contamination: $1.0 \times 10^{6}$ iu/ml. Both vectors were free of contaminating replication
competent adenovirus and lipopolysaccharides. The ability of both vectors to transduce and kill Lewis rat glioma cells(1) in the presence of GCV was confirmed in vitro (data not shown) before in vivo studies were performed. Both HC-Ad and Ad vectors were used at a dose of $1.5 \times 10^8$ vg/3ul delivered into the tumor (from bregma: +1 mm anterior, +3 mm lateral, and -5mm from the dura).

As the majority of patients undergoing gene therapy for glioma are likely to have a pre-existing immune response to adenovirus, we wished to test the effectiveness of both gene therapy vector platforms in an animal model that more closely mimics the immunological status that would be encountered in human GBM patients. To do so, we systemically immunized Lewis rats with a first generation Ad or saline (controls). Two weeks later animals were stereotactically implanted with the syngeneic Lewis rat glioma cell line CNS-1 into the striatum (1, 11). One week later, groups of animals received an intratumoral injection of Ad-TK, HC-Ad-TK, Ad-βgal, HC-Ad-βgal, or saline. GCV was administered systemically and animals were monitored for survival for 80 days. Survival was significantly improved in 75% of non-immunized animals treated with HC-Ad-TK and 60% of the animals treated with Ad-TK (*p<0.0001 vs. saline, log-rank test) (Fig 1, a). However, only the treatment with HC-Ad-TK significantly extended survival of pre-immunized rats (*p<0.0001 vs. saline, log-rank test). Treatment with Ad-TK failed to improve survival in pre-immunized animals (Fig 1, b). The ability of circulating anti-adenovirus antibodies from sera of immunized mice to neutralize both Ad and HC-Ad vectors was confirmed by a neutralizing antibody
assay (Fig 1, c) and the number of IFNγ-secreting lymphocytes was assessed by ELISPOT assay (Fig 1, d) (2).

To assess the efficacy and neuropathological consequences of gene therapy, we analyzed coronal brain sections of moribund, tumor bearing rats, or long-term survivors (day 80) by immunocytochemistry for markers specific for structural integrity and inflammation using markers for tyrosine hydroxylase (TH), myelin basic protein (MBP), CD8+ T cells (CD8), and macrophages/activated microglia (CD68) (2, 9, 10) (Fig 2). Coronal brain slices of five animals for each treatment group were examined and representative images are shown. Immunohistochemistry revealed hypertrophied activated astrocytes (GFAP+) surrounding the tumor or injection sites in all animals (Fig 2, a-e). Immunoreactivity for tyrosine hydroxylase (TH) (Fig 2, l-p) and myelin basic protein (MBP) (Fig 2, f-k) demonstrated that CNS-1 tumor growth displaces and compresses normal striatal brain tissue (TH+), as well as axons bundles (MBP+) coursing throughout the striatum. TH and MBP staining in long-term survivors showed no significant structural disturbances in the striatum when compared with the untreated, contralateral hemisphere, suggesting that brain tissue compression does not elicit irreversible neuropathological damage. Enlarged ventricles were observed in the hemisphere ipsilateral to tumor implantation in long-term survivors. Striatal CD8+ lymphocytes were observed throughout the tumors of all moribund animals, but were absent from the striatum of animals in which tumor regression had ensued in response to the therapy.
Macrophages/microglia (CD68) were detected throughout the tumor mass of moribund animals but were confined to the area surrounding the injection site (site of tumor implantation) in long-term survivors (not shown).

Robust HSV1-TK immunoreactivity in the brains of pre-immunized rats one week after intracranial delivery of Ad or HC-Ad vectors (Fig 3 a-d) confirms that neutralizing anti-Ad antibodies are not capable of blocking vector transduction of brain tissue by either vector (2, 19). Immunocytochemistry revealed that CD8+ T lymphocytes and macrophages/microglia (Fig 3 e-l) are restricted to the injection site indicating the safety of both vectors in immunized animals. Overt neuropathology was not observed.

Neutralizing antibody responses to viral capsid proteins are thought to curtail transgene expression upon virus readministration by binding to the virus and inhibiting target cell infection. However, T cell responses also play an essential role in the elimination of transgene expression from transduced cells. Our results indicate that antibodies do not block transduction of the brain, neither from first generation, nor from HC-Ad; this is supported by the fact that at early times post-infection there is expression from both vectors (Fig. 3) (3, 18, 19). This is likely due to the fact that antibodies cannot cross the blood brain barrier and access the brain, and thus remain unable to block Ad- mediated infection. However, once HC-Ad vectors infect brain cells they will not express any viral antigenic epitopes, whilst first generation Ad will express low levels of adenoviral proteins.
in infected cells. The slight reduction in expression from HC-Ad is limited by the transient presentation of adenoviral capsid derived antigenic epitopes on MHC-I of infected cells. Since those epitopes are derived from input HC-Ad virions, once input virions have been degraded, anti-adenoviral immune T cells do not have any adenovirally derived epitopes that they can recognize, thus transgene expression from HC-Ad remains stable.

In agreement with the efficacy data (Fig 1 b), HSV1-TK expression in the brain is persistent in pre-immunized animals injected in the striatum with HC-Ad-TK thirty days earlier (Fig 4 d). Expression of HSV1-TK, however, is greatly reduced in pre-immunized animals injected with Ad-TK (Fig 4 b). Infiltration of CD8+ lymphocytes in the brains of pre-immunized animals injected with Ad-TK was confirmed by immunocytochemistry (Fig 4 f). CD8+ immunoreactivity was restricted to the area surrounding the injection site in pre-immunized animals injected with HC-Ad-TK (Fig 4 h). Overt neuropathology was not observed (not shown). The robust expression of HSV1-TK when encoded within HC-Ad, even in the presence of a pre-existing anti-Ad immune response, further emphasizes the usefulness of the HC-Ad vector in clinical trials for GBM.

In summary, our data indicate that gene therapy using HC-Ad withstands the serious challenge so far imposed by pre-existing anti-adenoviral immunity. As this has been raised as an almost certain stumbling block to the implementation of clinically effective adenoviral-mediated gene therapy, our work indicates that
this challenge can be overcome in the context of a clinically relevant experimental GBM model. The implementation of gene therapy clinical trials for glioblastoma multiforme using HC-Ad vectors presents hope to improve the life expectancy and overall prognosis of these patients.

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**Author Contributions**

G.D.K. performed research (in vivo efficacy, Ad neutralizing antibody assays and gene expression analysis), analyzed data, contributed to the writing; A.G. performed research (in vivo analysis of gene expression in the brain, analysis of immune infiltrates), analyzed data, contributed to the writing; W.X performed
research (in vivo analysis of HC-Ad-TK mediated gene expression in the brain);
K.M.K. performed research (cloned and characterized the high HC-Ad plasmid),
analyzed data, contributed to the writing; MP, performed research ( molecular
characterization of HC-Ad-HSV1-TK and NAB assays), analyzed data; D.L.
performed research (ELISPOT assays), analyzed data; D.P. and P.N. performed
research (large scale up and purification of HC-Ad vectors), analyzed data;
P.R.L. and M.G.C. designed the experiments and conceptualized the work
described, analyzed the data and wrote the paper. All authors discussed the
results and commented on the manuscript.

**Competing Interests Statement**

The authors declare that they do not have any competing financial interests.


Figure Legends:

Figure 1. Treatment of a syngeneic intracranial glioma model with HC-Ad-TK induces tumor regression and long-term survival even in the presence of a systemic immune response against adenoviruses. Treatment with Ad-TK fails in pre-immunized, tumor bearing rats. (a) Log rank test of Kaplan Meier survival curves demonstrate that treatment of non-immunized animals bearing intracranial gliomas with either HC-Ad-TK or Ad-TK induce tumor regression and long-term survival while (b) only treatment with HC-Ad-TK is effective at inducing tumor regression and long-term survival tumor bearing animals which had been pre-immunized with adenoviruses. Treatment with Ad-βgal, HC-Ad-βgal, or saline alone fails (*p<0.0001 vs. saline, log-rank test, n=5-11). (c) Sera from immunized and non-immunized animals was tested with a neutralizing antibody assay to confirm the ability of circulating anti-adenovirus antibodies to neutralize both Ad and HC-Ad vectors (*p<0.01, two-way ANOVA, n=3-5). (d) An ELISPOT assay was performed to confirm the presence of a specific anti-adenovirus T cell response (*p<0.01, student’s t test, n=5). Splenocytes were stimulated with heat inactivated Ad vector (+) or without (-). Experiments were repeated at least twice, values represent the means +/- SEM.

Figure 2. Treatment of a syngeneic intracranial glioma with HC-Ad-TK results in minimal neurotoxicity in pre-immunized long term survivors. Immunocytochemistry was performed on coronal brain sections from five
moribund animals or long-term survivors with markers for (in order from top to bottom): astrocytes (GFAP, a-e), myelin basic protein (MBP, f-j), and tyrosine hydroxylase (TH, k-o). Representative images are shown. Immunolabeling reveals the eradication of the tumor (‘T’) in pre-immunized animals treated with HC-Ad-TK with minimal residual neurotoxicity. Scale bars = 1.0mm.

Figure 3. HC-Ad and Ad vectors mediate high levels of short-term HSV-1 TK expression in pre-immunized non-tumor bearing animals with minimal inflammation at one week post vector delivery. Animals were pre-immunized with a systemic injection of adenovirus or saline alone (non-immunized) and two weeks later injected intracranially with Ad-TK or HC-Ad-TK. Animals were euthanized one week post vector delivery and brains from five animals per group were analyzed by immunocytochemistry. (a-d). Representative images are shown. Low magnification (left panels) and high magnification (right panels) images of HSV1-TK immunoreactivity demonstrate high levels of HSV1-TK expression in animals treated with HC-Ad-TK or Ad-TK, regardless of immunization status. ICC reveals minimal CD8+ lymphocytes (e-h) and macrophages/microglia (i-l) in the brains of all animals.

Figure 4. HC-Ad-TK mediates high levels of persistent HSV-1 TK expression in pre-immunized non-tumor bearing animals with minimal inflammation at one month post-vector delivery. Animals were pre-immunized with a systemic injection of adenovirus and two weeks later injected intracranially with HC-Ad-TK
or Ad-TK. Animals were euthanized thirty days post vector delivery into the striatum and brains from five animals were analyzed by immunocytochemistry. Representative images are shown. Low magnification (left panels) and high magnification (right panels) images of HSV1-TK immunoreactivity demonstrate high levels of persistent HSV1-TK expression only in animals treated with HC-Ad-TK (d). Note the complete abrogation of TK expression in pre-immunized animals thirty days after injection with Ad-TK (b). ICC indicates high levels of CD8+ lymphocytes in pre-immunized animals injected with Ad-TK (f) while CD8+ lymphocytes are confined to the injection site in pre-immunized animals injected with HC-Ad-TK. Macrophages/microglia were observed in the brains of all animals (i-l).
Figure 1. King et al.

(a) Non-immunized

(b) Pre-immunized

(c) n.s.

(d) IFNγ spots

Neutralizing Antibody Titer

Skin: Pre-Imm./Ad, Saline/Ad

Brain: Pre-Imm./HC-Ad, Saline/HC-Ad

Ad: Pre-immunized +, Non-immunized -
Figure 2. King et al.
### Figure 3. King et al.

**HSV1-TK expression one week post-vector delivery**

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Figure 4. King et al.

HSV1-TK expression one month post-vector delivery

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High-Capacity Adenovirus Vector-Mediated Anti-Glioma Gene Therapy in the Presence of Systemic Antiadenovirus Immunity

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