Improved Adeno-associated Viral Gene Transfer to Murine Glioma

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Abstract

Glioblastoma (GBM) is a deadly primary brain tumor. Current treatment, consisting of surgical removal of the tumor mass followed by chemotherapy and/or radiotherapy, does not significantly prolong survival. Gene therapies for GBM are being developed in clinical trials, for example using adenoviral vectors. While adeno-associated virus (AAV) represents an alternative vector system, limited gene transfer to glioma cells has hampered its use. Here, we evaluated newly emerged variants of AAV capsid for gene delivery to murine glioma. We tested a mutant AAV2 capsid devoid of 3 surface-exposed tyrosine residues, AAV2 (Y444-500-730F), and a “shuffled” capsid (SH19, containing sequences from several serotypes) that had previously been selected for enhanced giall gene delivery. AAV2 (Y-F) and SH19 showed improved transduction of murine glioma GL261 cells in vitro by 2- to 6-fold, respectively, over AAV2. While AAV2 gene transfer to GL261 cells in established tumors in brains of syngeneic mice was undetectable, intratumoral injection of AAV2 (Y-F) or SH19 resulted in local transduction of approximately 10% of tumor cells. In addition, gene transfer to neurons adjacent to the tumor was observed, while microglia were rarely transduced. Use of self-complementary vectors further increased transduction of glioma cells. Together, the data demonstrate the potential for improved AAV-based gene therapy for glioma using recently developed capsid variants.

Keywords: Glioma; CNS, AAV; Gene transfer; Cancer

Introduction

The first decade of the 21st century has witnessed innumerable advances in the medical knowledge and practice. Yet there are multiple diseases eluding current treatments. Glioblastoma (GBM) is one of them [1,2]. Standard care for GBM, the most common and highly malignant adult brain pathology consists of surgical resection of the tumor mass followed by chemo and/or radiotherapy. The median survival for patients with GBM is 12-18 months. Due to its invasive nature, lack of a defined tumor edge and sensitive location complete removal of tumor tissue is virtually impossible. Furthermore, GBM is inherently difficult to treat partially due to the blood-brain barrier, and tumors easily develop resistance to both chemo- and radio-therapies [3,4].

Gene therapy as an alternative approach for glioma therapy has yielded encouraging results in preclinical studies and promising safety profiles in phase I/II clinical trials [5]. Therapeutic efficacy in phase III trials is currently being investigated [6]. Retroviral and adenoviral vectors for therapeutic/cytotoxic gene delivery for glioma treatment have been dominating the field. Oncolytic viruses (herpes simplex virus, adenovirus, reovirus, poliovirus, Newcastle disease virus and measles virus) have been used to a lesser extent.

Main avenues of anti-glioma genetic therapy are: 1. Conditional cytotoxic approaches, based on introduction of non-toxic enzyme into tumor cells which upon prodrug administration convert the prodrug into toxic compound capable of killing tumor (herpes simplex virus type-1 thymidine kinase (HSV1/Tk)/ganciclovir (GCV), cytochrome P450/CPA, cytosine deaminase (CD)/5-fluorocytosine (5-FC)) [7-9]; 2. Targeting toxins to the receptors overexpressed on glioma cells (IL13Ra2, the urokinase-type plasminogen activator (uPA) receptor, the epidermal growth factor (EGF) receptor, transferrin receptor) [10,11]; 3. Suppression of angiogenesis [12,13]; 4. Immune stimulation (enhancement of the immune response using cytokines such as GM-CSF, interleukins or interferons for T cell activation or dendritic cells mobilization) [14-16]; 5. Delivery of transgenes commonly mutated in glioma to correct the genetic alterations (P53/ARF/human MDM2, P16/Rb/cyclinD/CDK4, the receptor tyrosine kinase (RTK/Ras and PI3K/PTEN/Akt pathway) [17,18]; and 6. Combination approaches (e.g. HSV1/Tk-ganciclovir and fms-like tyrosine kinase-3 ligand, Flt3L) [19,20].

In pre-clinical studies, experimental murine models of GBM are utilized. These are either based on transplantable cell lines or represent genetically engineered animals. The former category can be subdivided into syngeneic GBM models generated by implantation of murine glioma cell lines in an immunocompetent mouse. Such models have been generated by modifying genes known to be altered in human gliomas (inactivating p53, PTEN, NF1 and/or overexpressing EGFR and/or PDGF, Flt3L). Gene targeted animal models have been generated by modifying genes known to be altered in human gliomas (inactivating p53, PTEN, NF1 and/or overexpressing EGFR and/or PDGF, Flt3L).

Recombinant adeno-associated virus (rAAV) has gained reputation as a safe and highly efficient gene therapy reagent in numerous disease models and in the clinical trials for Leber’s Conjunction Amusoria, hemophilia B, and aromatic L-amino acid decarboxylase deficiency...
and glioma cells in vitro. They found that AAV vectors with surface-exposed tyrosine modifications can reproducibly grow small tumor pieces on the syngeneic mouse strain, GL261, which was maintained by serial intracranial and subcutaneous transplantations of 3-methylcholanthrene into brains of C57BL/6 mice and human glioblastoma [47]. Initially produced by implantation of astronulate from the adult human cerebral cortex for enhanced gene transfer. AAV vectors have been shown to transduce astrocytes in the brain and have been primarily tested for neurodegenerative disorders (Parkinson disease, Alzheimer disease, Huntington disease, amyotrophic lateral sclerosis, Canavan disease) due to the high natural affinity of the viral capsid to neurons [30]. Attempts to utilize AAV for brain malignancies have shown promising results. Okada et al. demonstrated 35-fold reduction in the mean volume of tumors formed by U-251SP human glioma cells stereotactically injected into the brains of nude mice and treated with AAV-TK-1RS-IL2 vector injected into tumor coupled with ganciclovir (GCV) treatment. Several labs reported remarkably prolonged survival of mice with the same xenograft model after repeated injections of AAV-IFN-β into the tumor or after systemic delivery of IFN-β [33-35]. Of particular importance is the fact that AAV effectively penetrates solid tumors. For example, Enger et al. have compared AAV2 and adenovirus (Ad) 5 recombinant vectors on glioma spheroids in vitro and demonstrated distinct superiority of AAV2 over Ad5. This was also seen in vivo in human patient biopsies xenografted into nude rats and evaluated by MRI and histologic analysis [36]. In a follow up study, the same group tested alternative AAV serotypes, (AAV4 and AAV5) on five different glioma lines, spheroids generated from glioblastoma patient biopsies, and spheroid xenografts. Ultimately, AAV2 was found to be more efficient than the other serotypes [37-39]. In other work, AAV1 and AAV6 exhibited similar efficiencies compared to AAV2 [38]. AAV9 and AAV rh.10 serotypes have been shown to transduce astrocytes in the brain following peripheral delivery due to their ability to cross the blood-brain barrier [40]. Their utility for gene transfer to glioma cells remains to be investigated. Using in vitro studies, gene transfer to glioma cells based on variants derived from these vectors showed only minimal improvement over AAV2 [39].

In an effort to improve on AAV gene transfer to glioma, we set out to test two recently described AAV capsid variants. One is AAV2 devoid of 3 surface-exposed tyrosine residues, which are potential sites for phosphorylation, a known signal for ubiquitination [41-44]. This modified capsid shows reduced proteosomal degradation upon cellular entry, thereby enhancing translocation of the virus to the nucleus, which in turn increases transduction efficiency [45]. The second variant, ShH19, is a “shuffled” capsid based on AAV2 but with sequence elements from several other serotypes. This capsid had been obtained through repeated selection of a capsid library on primary astrocytes from the adult human cerebral cortex for enhanced gene transfer to glia [46]. The murine GL261 glioma model has histopathologic similarities to human glioblastoma [47]. Initially produced by implantation of carcogen 3-methylcholanthrene into brains of C57BL/6 mice and maintained by serial intracranial and subcutaneous transplantations of small tumor pieces on the syngeneic mouse strain. GL261 was adopted to grow in vitro and became a widely used syngeneic cell line with reproducible growth rates that allows an accurate knowledge of the site of the tumor after injection. Using the GL261 murine glioma model, we found that AAV vectors with surface-exposed tyrosine modifications or ShH19 capsid variants transfer genes more effectively than AAV2 to glioma cells in vitro and in vivo.

Materials and Methods

Viral vectors

Three variants of rAAV capsid were used for vector production: wild-type AAV2 (WT), AAV2-Y444-500-730F and ShH19, kindly provided by Dr. David Schaffer, University of California, Berkeley. The following transgenes were packaged: CBA-RFP, scCBA-GFP and CBA-Luc-mApple. Viral vectors were produced by the method of transfection of HEK-293 cells with liposomes. Liposomes were prepared from DOTAP and DOPE (Avanti Polar Lipids) in 2:1 ratio in the Rotavapor according to Avanti protocol. Viral particles were purified on step iodixanol gradients and concentrated in the Apollo 20 ml centrifugal concentrators [48]. Viral titers were determined by dot blot assay and validated by visualizing the capsids by Western Blot.

Cells

Murine GL261 glioma cells [47] were maintained in RPMI-1640 medium (Cellgro, Mediatech) supplemented with 10% FBS, 1% Penicillin/Streptomycin, 4 mM L-Glutamine and grown at 37°C with 5% CO₂. To generate GL261 stably expressing GFP, GL261 cells were transfected with pTR-UF5 (CMV-GFP) plasmid [49], and individual clones were selected in the presence of G418 (400 µg/ml). GFP/GL261 tumor cells had growth characteristics similar to the parent GL261 cells.

Animals

C57BL/6 mice (6-8 weeks old) were used in the study. CX3CR1(+/−) heterozygous C57BL/6 mice were as previously described [50]. In these mice all cells normally expressing CX3CR1 also express GFP. All procedures involving animals were carried out in accordance with the guidelines of the University of Florida Institutional Animal Care and use Committee (IACUC). GL261 glioma cells or GL261/GFP cells (1 × 10⁴) in a total volume not exceeding 3 microliters were injected 3 mm deep into the right cerebral hemisphere (2 mm posterior and 1.5 mm lateral from Bregma). Two weeks later 10° vector genomes were delivered directly into the tumor. Ten days after virus injections, animals were euthanized using sodium pentobarbital (32 mg/kg) and subsequently perfused with saline and ice-cold 4% PFA in 0.1 M Phosphate buffer (PB), pH 7.4.

Tissue processing

Following perfusion brains were removed and postfixed for 4 hours, then tissues were incubated in 30% sucrose solution at 4°C overnight followed by freezing in 2-methylbutane at -42 to -45°C for 1 to 2 min and stored at (-80°C) until sectioned. Frozen whole brains were cut into 40-micron sections using a Leica CM 3050S freezing microtome and collected in Walter’s anti-freeze solution. The sections were washed in PBS/0.9%NaCl/0.25% Triton x-100, some processed for immunohistochemistry and mounted onto Superfrost Plus microscope slides [51]. For each brain approximately 100 sections were analyzed (10 slides × 10 sections per slide). The sections were examined using Leica AF6500 confocal laser-scanning microscope. Sequential scanning was used to suppress optical cross talk between the fluorophores in stationary structure colocalization assay. Adjustments of contrast and illumination were made utilizing Adobe Photoshop CS software.

Results and Discussion

Alternative AAV capsids improve in vitro transduction of murine glioma cells

Recombinant Adeno-associated vectors (rAAV) expressing Red Fluorescent Protein (RFP, mApple) under control of CMV enhancer/chicken b-actin promoter (CBA) were generated: rAAV2-WT-CBA-RFP, rAAV2-TRP-CBA-RFP and rAAV-ShH19-CBA-RFP. Triple-mutant rAAV2-TRP (Y444+500+730F) is a capsid variant of AAV.
serotype 2 in which tyrosine residues at positions 444, 500 and 730 are substituted with phenylalanine (Y-F). Tyrosine phosphorylation of intact AAV particles by epidermal growth factor receptor tyrosine kinase (EGFR-PTK) interferes with intracellular trafficking of the virus from cytoplasm to the nucleus by tagging it for ubiquitination and proteasome-mediated degradation thus significantly reducing therapeutic potential of the vector. Replacement of tyrosine residues with phenylalanine can substantially improve transduction of various cell types, such as hepatocytes, fibroblasts, retinal cells and mesenchymal stem cells, among others, both in vitro and in vivo [41,45,52]. rAAV-ShH19 is a product of molecular evolution for transduction of astrocytes (which increased efficiency 5.5-fold compared to parental rAAV2) [46]. Vectors based on both capsid variants alongside with rAAV2-WT, which has been shown to be a relatively better serotype in different glioma cell lines (U87-MG, A172, D37, GaMG, HF-66, U373 [32-34]) were used to infect GL261 cells in vitro. Infection was done at three different MOIs: 100, 1000, and 10,000. At the highest MOI, ~1.4% of AAV2, ~2.5% of AAV2-TRP and ~8.5% of AAV-ShH19 infected cells tested RFP positive by flow cytometry (Figure 1). Use of an MOI of 10,000 for in vitro transduction was in the range reported by others for different glioma cell lines [38,39,53]. Both rAAV2-TRP and rAAV-ShH19 demonstrated higher transduction efficiency for GL261 cells in vivo compared to rAAV2-WT (2- and 6-fold, respectively).

Alternative AAV capsids substantially improve in vivo transduction of murine glioma cells

Due to the fact that glioma tumors are highly necrotic (and tumor affected brain sections are extremely fragile), our strategy for in vivo evaluation was to minimize post viral injection manipulation and utilize systems for detection of co-localization of gene transfer with glioma cells. Thus, we generated GL261 cells stably transfected with a GFP reporter. GL261-GFP cells had growth characteristics similar to the parental cell line and formed tumors in the recipient animals in predictable manner. For all experiments, 1×10^5 GL261 or GL261-GFP cells were injected stereotaxically into the brain of C57BL/6 mice.
followed by intratumor injection 13 days later with 1×10⁹ vg in 1-2 µl of vector solution. Initially, three virus vectors, rAAV2-WT-CBA-RFP, rAAV2-TRP-CBA-RFP and rAAV-ShH19-CBA-RFP were tested in healthy mouse brain or in parental GL261 tumor-bearing brain (not marked by GFP). RFP expression could be easily detected 7 days post gene transfer, with no further increase by day 10; while no transgene expression was observed at day 3 (data not shown). Therefore, we proceeded with in vivo gene transfer to GL261-GFP brain tumors in C57BL/6 mice, followed by tissue analyses 7-13 days later. As shown in figure 2, RFP expression was easily detected in the tumor from all vectors tested (at least 4 animal per vector were analyzed; 3-4 slides with tumor-containing brain sections/animal; 10 sections/slide). However, only rAAV2-TRP (Figures 2B and 2E) and rAAV-ShH19 (Figures 2C and 2F) vectors transduced GL261 glioma cells as judged by co-localization of RFP delivered by the virus and GFP expressed by glioma cells (yellow/orange in representative panels). Within the area of the tumors positive for transgene expression, the efficiency of transduction of glioma cells was approximately 10% for both capsid variants as quantified using Leica AF6500 software. Presence of additional exclusively red fluorescent cells indicate a level of viral transduction of non-glioma cells within the tumor. In order to achieve more widespread transduction throughout the tumor, improvements in the injection/delivery technique, vector formulation, and perhaps further modification of the capsid sequence are required.

In vivo transduction is not restricted to glioma cells

In addition, rAAV2-TRP and rAAV-ShH19 vectors (but not rAAV2-WT) transduced neurons at the edge of the tumor (Figure 3A; while not labeled by specific antibody, these RFP expressing cells had characteristic morphologic appearance of neurons as shown more clearly in a representative example in Figure 3B). In normal brain, AAV2 is known to predominantly target neurons and epithelia within the central nervous system due to the receptor-based tropism, as heparan sulphate proteoglycan (HSPG) is prevalent on neuronal cell surfaces [54]. When tested in the healthy (non-tumor baring) brains, rAAV2-TRP and rAAV-ShH19 retained the ability to transduce neurons and showed a larger spread of transduction from the site of the injection than what was observed for rAAV2-WT (Figure 4). This may explain the spread of viral transduction for these two capsids beyond the tumor, reaching the layer of neurons outlining the tumor. In contrast, the traditionally reported low spread of rAAV2 in the brain may have restricted rAAV2-WT distribution and prevented neuronal binding away from the intratumor injection site. Thus, capsid variants rAAV2-TRP and rAAV-ShH19 demonstrated a substantial
GFP-positive microglia are present in significant numbers within gliomas. Using these mice, we have established that the expression of MHC class I and II molecules, production of powerful immunosuppressive factors such as TGF-β, down-regulating immune responses, which may be elicited against the tumor [56]. Immune stimulation aimed at the patient’s immune system to destroy tumor cells is one of the strategies of gene therapy for glioma [55]. Microglia are the main resident immunological cells of mesodermal origin in the CNS. In normal brain, AAV2 does not transduce murine microglia [57]. There have been reports of in vitro transduction of primary rat microglia at high MOI (10,000) [58,59]. Improving gene transfer to microglia would be desirable goal of gene therapy for glioma.

Mice heterozygous for CX3CR1 deficiency (C57BL/6(CX3CR1<sup>+/−</sup>)) were used for these experiments. We have established that GFP-positive microglia are present in significant numbers within intracranial GL261 tumors [60]. Therefore, co-localization of RFP expressed from the vector and GFP present in microglia would indicate microglia transduction. All three vectors only rarely transduced microglia in healthy brain or within the tumor (Figure 5A). Besides “true” co-localization events of the cytoplasmic gene products (RFP delivered by the virus and GFP transgenically expressed in microglia), indicating transduction, we also observed red and green fluorescence located in different compartments of the same cell without co-localization (Figure 5B). Similar observations have been reported for uptake of fluorescently labeled rAAV2-Cy3 virus by microglia [57]. We speculate that activated microglia, faithful to their role as antigen presenting cells, might have engulfed virus-infected cells.

**Use of scAAV vectors further improves glioma cell transduction**

Although AAV capsid mutants increased transduction efficiency of glioma over rAAV2-WT, further improvement of efficiency is desirable. Utilization of the self-complementary (double-stranded) genomes in AAV vectors circumvents second-strand DNA synthesis or complementary strand recruitment limitations [61] and increases the effective dose of the vector [62]. In order to assess whether this strategy could be useful for glioma transduction, we performed in vitro infections of GL261 cells using rAAV2-WT-scCBA-GFP, rAAV2-TRP-scCBA-GFP and rAAV-ShH19-CBA-GFP self-complementary vectors. As shown in figure 6, at a low MOI of 500 vg/cell, ~32% of the rAAV2-WT, ~48% of the rAAV2-TRP and ~86% of the rAAV-ShH19 vectors infected cells tested GFP positive by flow cytometry. Nearly identical results were obtained in a second independent experiment, where GFP+ cells were manually counted (data not shown). Therefore, use of scAAV vectors should further increase efficacy but is limited to small transgenes that fit into the packaging limits for self-complementary genomes (<3 kb). The increase of in vitro transduction efficiency by using the scAAV vector was remarkable (~2-log). It is possible that the
comparatively low fluorescence intensity of the RFP transgene product caused us to underestimate the in vitro performance of the ssAAV vectors. Nonetheless, transduction with scAAV was substantially more robust, which warrants future in vivo studies with these vectors.

In summary, two capsid-modified variants of rAAV2 have been tested for gene delivery to GL261 mouse model of glioma. Both rAAV2-TRP and rAAV-ShH19 mutants demonstrated superior transduction of GL261 cells in vitro and in vivo. Further improvement of in vivo gene transfer may be required, depending on the therapeutic strategy, which may involve use of higher vector doses, scAAV vectors, improved delivery techniques, and further optimization of the viral capsid. Transduction of neurons in the vicinity of tumor cells may be an advantage or an undesired effect for the therapy, depending on the strategy and therapeutic mechanism.

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Author Statement


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