Biochemical and Biophysical Research Communications 482 (2017) 506–513

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Gene delivery of apoptin-derived peptide using an adeno-associated virus vector inhibits glioma and prolongs animal survival

Xiuli Zhong [a, 1], Hengyu Zhao [b, 1], Songhe Liang [a], DanYang Zhou [a], Wenjia Zhang [b], Lijie Yuan [a, *]

[a] Department of Biochemistry and Molecular Biology, Daqing Campus, Harbin Medical University, Daqing, Heilongjiang, 163319, PR China
[b] Daqing Oilfield General Hospital, Daqing, PR China

ARTICLE INFO

Article history:
Received 10 August 2016
Received in revised form
13 October 2016
Accepted 18 October 2016

Keywords:
AAV2
HSP70
Apoptin
Glioma
Apoptin-derived-peptide (ADP)

ABSTRACT

Glioblastoma (GBM) is the most common malignant brain tumor in adults. We designed an adeno-associated virus (AAV) vector for intracranial delivery of the secreted HSP70-targeted peptide APOPTIN derived from Apoptin to GBM tumors. We applied this therapy to GBM models using human U87MG glioma cells and GBM xenograft models in mice. In U87MG and U251MG cells, conditioned medium from AAV2-apoptin-derived peptide (ADP)-expressing cells induced 83% and 78% cell death. In mice bearing intracranial U87MG tumors treated with AAV2-ADP, treatment resulted in a significant decrease in tumor growth and longer survival in mice bearing orthotopic invasive GBM brain tumors. These data indicate that ssAAV2-ADP injection in the left hemisphere effectively prevented ipsilateral tumor growth but was insufficient to prevent distal tumor growth in the contralateral hemisphere. However, the systemic route is the most effective approach for treating widely dispersed tumors. In summary, systemic delivery of AAV2-ADP is an attractive approach for invasive GBM treatment.

1. Introduction

Glioblastoma multiforme (GBM) is classified as WHO grade 4 astrocytic glioma and is the most common and aggressive type of primary brain tumor [1–3]. Although chemotherapy, radiotherapy and surgery are used clinically to treat GBM, the median survival time of patients with gliomas is only 13–16 months [4]. Thus, there is a strong unmet medical need for the development of novel therapies with improved clinical efficiency and enabling longer survival times in GBM patients.

Gene therapy, defined as the introduction of nucleic acid polymers into a host cell as a source of new drugs, is mainly used for the treatment of serious diseases and is very promising for the treatment of tumors. More than 2000 clinical trials employing gene transfer have been performed and generally established that a number of vehicles or vectors are safe [24,25]. To increase the success of cancer gene therapies, however, a major hurdle must be overcome: the development of gene delivery vectors that can efficiently, safely and specifically deliver genetic material to a patient's cells. In particular, AAV vectors are increasingly successful due to their gene delivery efficacy, lack of pathogenicity, and strong safety profile and are among the most commonly used viral vectors for gene therapy. Based on these properties, AAV vectors have enabled clinical successes in a number of recent clinical trials that have established the promise of gene therapy in general, including for the treatment of diseases such as Leber's congenital amaurosis (LCA) [10–12]. AAV vectors with excellent gene delivery properties have been harnessed for cancer studies in vitro, cancer preclinical models in vivo, and more recently, cancer clinical trials under development, such as vectors based on adeno-associated virus serotype 2 (AAV2). These vectors have been used as effective vectors for delivering therapeutic genes to the tumor and effectively suppressing the growth of malignant gliomas in the brains of mice [13]. We previously demonstrated that an AAV vector mediated the co-expression of IL-24 and apoptin and significantly suppressed hepatocellular carcinoma cell growth both in vitro and in vivo [14].

Apoptin, a 14-kDa viral protein (chicken anemia virus protein-3,VP3), selectively induces apoptosis in cancer cells [5,6]. Apoptin-mediated cell toxicity depends on its cellular localization; nuclear localization promotes cell death, whereas cytoplasmic localization does not [7,8]. The 70-kDa heat shock protein (HSP70)

* Corresponding author.
E-mail address: yuanlijie1225@163.com (L. Yuan).
[1] Xiuli Zhong and Hengyu Zhao contributed equally to this study.

http://dx.doi.org/10.1016/j.bbrc.2016.10.059
0006-291X/

facilitates the folding, assembly, transport and degradation of biological macromolecules. HSP70 inhibits the release of cytochrome C, thus preventing induction of apoptosis by procaspase-9, which is highly expressed in tumor tissue and poorly expressed in normal tissues. Thus, HSP70 is considered a molecular target of tumors. Apoptin directly binds the promoter of HSP70 and inhibits HSP70 transcription [26]. We aimed to promote the apoptosis-inducing capacity of apoptin in tumor cells using a polypeptide sequence of reduced length. Thus, we created an apoptin-derived-peptide (ADP) to target the promoter region of HSP70. The HSE-targeting ADP that we designed comprised four parts: a TAT tag, the core sequence of NLS1, the LRS, and NLS2. With this design, the LRS creates a flexible connection between the two NLSs to effectively maintain the necessary three-dimensional structure. We demonstrated that compared to apoptin, ADP more strongly promotes tumor cell apoptosis, reduces tumor growth, improves animal survival and inhibits glioma invasion and migration in vivo.

To assess the possibility of glioma gene therapy using an AAV vector expressing ADP, we performed both in vivo and in vitro experiments by inhibiting human glioma growth in the brains of nude mice and analyzing cultured U87MG glioblastoma cells. Our results demonstrated that ADP gene transfer effectively suppressed U87MG brain glioma growth in mice and that systemic delivery of an AAV2-ADP vector may be effective for GBM treatment.

2. Materials and methods

2.1. Cell culture

The human GBM cells lines U87MG and U251MG and human embryonic kidney 293 cells were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were routinely cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum.

2.2. Plasmids

To construct an AAV expression vector, Apoptin-Derived-Peptide cDNA was amplified from I.M.A.G.E. by PCR using the primers 5′-TTTCAATTGACCTATGGCCGTAAAAAACGTCGT-3′ and 5′-TTTCAATTGACGACGACGCTGACGACG-3′, which contain *Mun*I sites (*Eco*RI-compatible cohesive ends). The PCR products were digested with *Mun*I and inserted into the AAV plasmid pCAaGBE, which encodes the CAGGS promoter and the green fluorescent protein (GFP) gene driven by the B19 promoter. The resultant recombinant plasmids were named pAAV2-ADP. The AAV-apoptin-specific method was described previously [9]. The AAV vector stocks were generated using an adenovirus-free triple-transfection method.

2.3. AAV vector transduction

U87MG or U251MG cells were seeded in 96-well plates at a density of 5×10^3 cells/well in complete DMEM medium. Infections were performed with a multiplicity of 1×10^3 or 1×10^4 viral genomes/cell in serum- and antibiotic-free DMEM medium. The expression of EGFP in the infected cells was analyzed by direct fluorescence imaging using a Zeiss Axiovert 25 fluorescent microscope. Representative images were taken with a × 20 objective. The viral transduction efficiency was quantified by ImageJ (NIH, Bethesda, MD). Three visual fields were analyzed for each experiment.

2.4. Cell viability assay

U87MG or U251MG cells were seeded into 96-well plates and infected with AAV 16 h later. One to 5 days after infection, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) was added to each well, and the plates were incubated at 37 °C for 4 h. The medium was then removed, and 100 μl of 0.04 M HCl-isopropanol was added to each well. The absorbance was measured at 595 and 655 nm using a microplate reader (Molecular Devices, USA). For each assay, ten replicate wells were counted.

2.5. ELISA

The concentration of TAT (HOVERcruz, USA) in conditioned medium from U87MG cells was determined by ELISA in 96-well plates using standard techniques. Briefly, the plates were coated with a polyclonal Ab (I) against TAT (HOVERcruz, USA) overnight at 4 °C in standard sodium carbonate coating buffer, followed by blocking for 2 h at room temperature in blocking buffer (PBS containing 1% bovine serum albumin (BSA) and 1% thimerosal). The samples or recombinant TAT were diluted in diluent buffer (blocking buffer with 1% Tween 20), added to the plates, and incubated for 2 h at room temperature in diluent buffer containing 2% nonfat dry milk. After extensive washing with 0.1% Tween 20 in PBS, a biotinylated polyclonal Ab (II) against TAT was added to the plate, and the plate was incubated for 1 h at room temperature. After washing, horseradish peroxidase (HRP)-streptavidin (Amersham Life Science; Shanghai, China) was added to the plates, and the plates were incubated for 30 min at room temperature. The reaction was developed by the addition of peroxidase substrate and was stopped with NH_2SO_4. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices, USA).

2.6. Western blot analysis

U87MG cells were seeded in six-well plates at a density of 5×10^5 cells/well and treated as indicated. Equal aliquots of the lysates from the cells were subjected to 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5% bovine serum albumin (Sigma) for 1 h and then probed with the following primary antibodies at 4 °C overnight: caspase-3; caspase-9 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Next, the membranes were washed and incubated with an HRP-conjugated secondary antibody at 1:5000 for 2 h. The membranes were developed by enhanced chemiluminescence kit (Beyotime, China) following the manufacturer's protocol.

2.7. Apoptosis assay

U87MG cells were infected with AAV as indicated and then seeded into 12-well plates. After culturing for the indicated time, the cells were incubated with 5 μl of annexin V-allophycocyanin conjugate (annexin V-APC) and 5 μl of 7-amino-actinomycin D (7-AAD) (BD Biosciences) for 15 min in the dark at room temperature. The samples were subjected to flow cytometry on a BD FACSCanto II (Becton Dickinson), and 3×10^4 cells were analyzed per sample. The data were analyzed using FlowJo software.

2.8. Immunofluorescence and fluorescent imaging

AAV2-ADP or AAV2-Apoptin and AAV2-EGFP were added to adherent cells cultured in DMEM for 12 h. After culturing for 24 h, the cells were fixed in 4% paraformaldehyde in PBS, permeabilized in 0.1% Triton X-100, and stained with either a cytochrome *c* antibody (Invitrogen, USA) or a TAT antibody, followed by the respective Cy3-conjugated secondary antibodies. Next, 20 μl of Hoechst 33342 nuclear dye was added to each well, and after 20 min, the