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A non-viral suicide gene delivery system traversing the blood brain barrier for non-invasive glioma targeting treatment

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ABSTRACT

Herpes simplex virus type I thymidine kinase gene (HSV-TK) in viral vector is a promising strategy against glioblastoma multiforme (GBM). However, the biosafety risk restricts its application in clinic. In this work, poly (L-lysine)-grafted polyethylenimine (PEI-PLL), which combines the high transfection efficiency of polyethylenimine and the good biodegradability of poly (L-lysine), was adopted as the non-viral vector backbone. Angiopep-2, a blood brain barrier (BBB) crossing and glioma targeting bifunctional peptide was conjugated on PEI-PLL via polyethyleneglycol (PEG) and designated as PPA. The optimal transfection ratio of PPA/DNA complexes nanoparticles (PPA NPs) was firstly characterized. Next, the glioma targeting of the PPA NPs was confirmed through cellular uptake and transfection analysis. The in vivo imaging studies demonstrated that the PPA NPs could not only penetrate BBB but also accumulate in striatum and cortex via systemic administration. Moreover, the PPA/HSV-TK NPs showed remarkably anti-glioma effect and survival benefit in an invasive orthotopic human GBM mouse model through inhibiting proliferation and inducing apoptosis ($p < 0.05$ vs control). This study firstly illustrated that the cationic polymer PPA could be exploited as an efficient gene vector to cross the BBB, and innovatively provided a potential non-viral nanomedicine for noninvasive suicide gene therapy in the glioma treatment.

1. Introduction

Every year, about 7 of 100,000 people worldwide are diagnosed with primary brain cancers, of which nearly 80% are malignant glioma [1]. In spite of the advances of the intracranial surgical resection, radiotherapy, chemotherapy and gene therapy in the past few decades, patients suffering from glioma still only have a median survival of <2 years after first diagnosis [2]. The high lethality rate of the glioma is due to the concerted action of manifold factors. For instance, the invasive and aggressive nature of glioma makes the complete surgical removal difficult. In addition, compared with other types of tumors, the shield of the blood brain barrier (BBB) could significantly inhibit the intracerebral delivery of the chemotherapy drugs and gene therapeutic agents.

The BBB is primarily formed by the specialized brain microvascular endothelial cells (BMEC) characterized by tight junctions (TJs) [3]. This physical barrier could maintain homeostasis between the blood compartment and the brain. Nearly 100% of large-molecule pharmaceutics and 98% of small molecules cannot cross the barrier [4]. This physiological mechanism is like a double-edged sword. On the one hand, the selective permeability makes the BBB function as the bastion to protect the delicate parenchyma from impairment of the adverse extraneous material. On the other hand, the BBB presents an uncompassable obstacle to the agentia for glioma patients [5]. Therefore, pursuing the effective strategies to enhance the intracerebral delivery of the therapeutic agent is a difficult but significant medical mission. In general, there are three possible strategies to overcome this dilemma. The first approach is circumventing the BBB, *i.e.*, direct intracerebral injection or implantation of the drug-releasing vector in the brain [6]. However, the inaccurate location of the focus, slowly drug diffusion in the brain, and the invasive physical injury all restrict the applications of these strategies in the clinic. The second method is elevating the permeability of the BBB *via* hypertonic chemical or physical approaches. Hypertonic mannitol can induce osmotic pressure between the vascular walls, break the TJs, and increase the BBB penetrability. However, it may cause permanent neurotoxicity due to the lack of specific targeting. Focused ultrasound treatment could locally break the BBB through the microbubble effect. Whereas, this strategy would also result in the irreversible BBB damage [7]. The third approach is the receptor mediated transcytosis (RMT) strategy, which can enhance the permeability of the BBB in the noninvasive glioma treatment. RMT is a biological process through which the endogenous molecules cross the BBB, owing to the expression of peptide-specific receptors in the membrane of the BMEC. The specific ligand can bind its corresponding receptor and

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induce the transcytosis event through the endocytic vesicles. Taking advantage of this property, receptor-targeting ligands such as transferrin, insulin, rabies virus glycoprotein peptide, and cyclic arginine-glycine-aspartic tripeptide have been extensively modified to the agentia to function as the molecular Trojan horse, which elevate the penetrability of the antibody, chemotherapeutics or the polymer/gene nanoparticles [8–10].

Angiopep-2, a 19 amino acid peptide derived from the Kunitz domain of the low density lipoprotein receptor-related protein 1 (LRP1) ligands, possesses a much higher BBB transcytosis efficacy than other peptide such as transferrin and its mother molecule aprotinin [11]. Furthermore, LRP1 receptors are expressed not only in BMEC, but also in many subtype of glioma [12]. Therefore, the modification of angiopep-2 could increase the BBB permeability and glioma targeting at the same time [13]. However, there are still two insurmountable flaws for the angiopep-2 modified drug or cationic gene carrier in the systemic administration. Firstly, the small molecule chemotherapy drugs or gene vector could be easily identified by the reticuloendothelial system (RES) in the blood circulation. Although the PEG modification can reduce this phenomenon, previous reports illustrate that the drug or gene concentration in the RES organs are still much higher than it in the brain, and this will inevitably increase the side effect of the chemotherapy drugs and genetic material to the non-tumor cells in normal tissue [14]. In addition, angiopep-2 modified carrier would suffer a great loss in the circulation, and only a small percentage could accumulate in the brain lesion area. The lower drug concentration or gene transfection efficiency severely limits the anti-tumor effect in the noninvasive glioma treatment [15]. Above study suggests that it would be reasonable and efficient to seek a breakthrough from the therapeutic agent itself: harmless to the normal cells, and only a modest dose could induce a powerful therapeutic effect to the glioma cells.

Suicide gene therapy, especially the herpes simplex virus type I thymidine kinase/ganciclovir (HSV-TK/GCV) system is one of the most commonly used gene therapy strategy against malignant glioma in preclinical and clinical studies [16,17]. Except for the selective cytotoxicity to actively dividing cancer cells transfected with HSV-TK, another attractive aspect of this system is the so-called by-stander effect, which could transfer cytotoxicity from the transfected cells to neighboring non-transfected cells and then profoundly enhance the anti-tumor effect [18]. HSV-TK/GCV system has been previously utilized for glioma therapy in viral vectors such as non-replicating herpes virus and adenovirus. However, the biosafety risk, small gene capacity, and inferior targeting restrict the regularly application of viral vectors in the clinic. On the other hand, due to the weak transfection efficiency *in vivo* and the blocking effect of the BBB, only one research has been carried out to utilize poly (β-amino ester) as the non-viral vector for HSV-TK plasmid through the direct intracerebral injection in orthotopic glioma mouse model [19]. Thus, it is still challenging and meaningful to establish a high efficient and less neurological harmful noninvasive administration strategy for the non-viral vector/HSV-TK system in primary glioma therapy.

Polyethylenimine (PEI) is one of the most widely utilized and studied synthetic polymer for gene delivery. The cationic PEI could efficiently condense the electronegative nucleic acids by electrostatic interaction. However, the non-degradability restricts the application of PEI *in vivo* [20]. As another common transfection material, poly (L-lysine) (PLL) could not condense DNA as effective as PEI, but prevails because of its biodegradability [21]. In order to maximize the advantages of these two non-viral gene vectors, poly (L-lysine)-grafted polyethylenimine (PEI-PLL) copolymer has been established and utilized as an efficient gene vector in human cervical and breast cancer cells [22,23]. The modification of PLL would significantly decrease the cytotoxicity of PEI-PLL. However, this cationic vector could only be utilized through *in situ* gene delivery systems *in vivo* because of the nonstability during the blood circulation [23,24]. In this work, a systemic delivery system was developed by modifying the PEI-PLL with angiopep-2 through the bifunctional polyethyleneglycol (PEG) (Scheme 1). Firstly, the angiopep-2 grafting could increase the BBB penetrability and the transfection efficiency in glioma cells by RMT simultaneously [25]. In addition, the PEG linker not only minimized the steric hindrance effect of the hyper-branched PEI-PLL to the targeting ligands, but also created a hydrophilic protective layer around the cationic vector. This modification could prevent the non-selective absorption of proteins in circulation *via* steric repulsion forces and make the PEI-PLL-PEG-angiopep-2 (PPA)/DNA nanoparticles (NPs) unrecognizable by the RES *in vivo* [26]. The optimal PPA/DNA NPs formulation for transfection was firstly characterized. Next, it was demonstrated that this non-viral vector could specifically target the glioma cells and deliver the reporting gene to traverse the BBB both *in vitro* and *in vivo* evaluation. At last, the antitumor effect and mechanism of the PPA/HSV-TK NPs *via* noninvasive administration was confirmed in an invasive orthotopic human glioma multiforme (GBM) mouse model (Scheme 1).

2. Material and methods

2.1. Reagents and materials

Branched PEI (Mw = 25 kDa) was purchased from Sigma (USA). PEI-PLL was prepared as previously reported. Angiopep-2 (TFFYGGSRGKRNNFKTEEYC) was acquired from Chinese Peptide Company (Hangzhou, China). MAL-PEG5k-NHS (Mw = 5 kDa) was bought from JenKem Technology (Allen, TX, USA). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). Dulbecco's Modified Eagle Medium (DMEM), RPMI1640 and fetal bovine serum (FBS) were purchased from Gibco (Grandland, NY, USA). Ultroser™ Serum Substitute was purchased from Pall (USA). Cy5-labeled DNA was obtained from RiboBio (Guangzhou, China). Luciferase (LUC) reporter gene assay kit and cell lysate were acquired from Promega (Wisconsin, USA). The BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). 4′, 6-diamidino-2-phenylindole (DAPI) (Roche, Mannheim, Germany) was used to stain the nuclei. Primary antibody included anti-TK (Santa Crus, CA, USA), anti-Ki-67 (Thermo, Rockford, IL, USA), anti-cleaved-caspase-3 (CST, Boston, Massachusetts, USA), and anti-α-tubulin (Sigma). Secondary antibody HRP-conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG were also purchased from Sigma. Terminal nucleotidyl transferase mediated nick end labeling (TUNEL) kit was purchased from Roche. The plasmid of PLOX-GFP-iresTK (TK) was purchased from Addgene (plasmid 12243). The plasmid encoding firefly luciferase (pGL3) was purchased from Promega.

2.2. Synthesis and characterization of PPA

PEI-PLL was obtained through the ring-opening polymerization reaction of Lys (*Z*)-NCA by PEI in anhydrous chloroform solution. The feed ratio of PEI and Lys (Z)-NCA unit (1/81) was in accordance with our previous work. This ratio showed better transfection efficiency and lower cytotoxicity than other ratios [22,23]. It has been generally demonstrated that PEG modification could increase the circulation time of the NPs. However, this augment has a limitation. The excess amount of PEG could not decrease the protein adsorption further [26]. On the contrary, when the molar ratio of PEG (Mw = 5 kDa)/PEI (Mw = 25 kDa) increased to 6/1 or 15/1, the polymer/DNA NPs would form into a less compact structure [27]. Previous research has proved the PEG could act as a linker between the PEI backbone and the glioma-targeting molecule, and its grafting density was 3:1 (PEG/PEI, mol/mol). This PEG modified gene vector has been proved to possess efficient gene transfection ability and obvious anti-glioma capacity with the therapy gene [9]. In this consideration, PEI-PLL was further reacted with MAL-PEG-NHS (PEG) at the ratio of 3:1 (PEG/PEI, mol/mol) in phosphate buffered saline (PBS, pH 8.0) for 4 h. The product, PEI-PLL-PEG (PP) was purified by ultrafiltration (molecular weight cutoff =