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Co-delivery of TRAIL gene enhances the anti-glioblastoma effect of paclitaxel *in vitro* and *in vivo*

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ABSTRACT

Co-delivery of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and paclitaxel (PTX) is an attractive strategy to enhance their anti-tumor efficacy. As the most aggressive brain tumor, glioblastoma is sensitive to TRAIL and PTX. However, their therapeutic efficacy for intracranial glioblastoma is significantly impaired by blood–brain barrier (BBB) and blood–tumor barrier (BTB). Previously, we have prepared c(RGDyK)–poly(ethylene glycol)–polyethyleneimine (RGD–PEG–PEI) as a non-viral gene carrier for glioblastoma targeted therapy by employing a cyclic RGD peptide (c(RGDyK), cyclic arginine–glycine–aspartic acid–D-tyrosine–lysine), which binds to integrin $\alpha_v\beta_3$ over-expressed neovasculature and U87 glioblastoma cells with high affinities. In the present work, it was found that low concentration of paclitaxel (10 nM) significantly enhanced the gene transfection of RGD–PEG–PEI/pDNA nanoparticle, which, in turn, dramatically elevated the anti-glioblastoma effect of paclitaxel *in vitro*. The gene transfection was also elevated *in vivo*. Co-delivery of brain-targeted CDX–PEG–PLA–PTX micelle dramatically enhanced gene transfection efficiency in the intracranial brain tumor. Due to the change of BBB integrity and the formation of BTB, we subsequently investigated the anti-glioblastoma effects of RGD–PEG–PEI/pORF-hTRAIL nanoparticle combined with CDX–PEG–PLA–PTX micelle (paclitaxel loaded CDX–poly(ethylene glycol)–*block*-poly(lactic acid) micelle). While at the same dosages, the median survival of the intracranial glioblastoma-bearing model mice treated with co-delivery (33.5 days) is significantly longer than those of solely treated mice with CDX–PEG–PLA–PTX (25.5 days), RGD–PEG–PEI/pORF-hTRAIL (24.5 days) or physiological saline (21.5 days). Herein, we verify the high potency of co-delivery of TRAIL gene and paclitaxel in the intervention of intracranial glioblastoma by employing tumor-targeted gene carrier RGD–PEG–PEI and brain-targeted micelle CDX–PEG–PLA, respectively.

1. Introduction

Treatment of glioblastoma multiforme (GBM), one of the most frequent primary malignant brain tumors (approximately 40%) [1], remains a challenge irrespective of the recent improvements. The integrity of blood–brain barrier (BBB) and blood–tumor barrier (BTB) hampers the tumor penetration and uptake, making it particularly inefficient of mostly therapeutic agents for GBM [2–4]. Even though BBB integrity is compromised during the development of tumors, it still influences the therapeutic efficacy in systemic administration [5,6].

At the early stage of GBM, tumor neovasculature has not formed and the glioma cells make use of the normal brain vessels [7]. With the progression of GBM, vasculature around and in GBM exhibits a wide range of permeability, from normal capillaries with essential no BBB leakage to a tumor neovasculature that freely passes even such large molecules as albumin [8]. Thus, brain-targeted drug delivery has much necessity, especially in the early stage of GBM, to circumvent BBB. Receptors highly expressed on the capillary endothelium of the brain such as nicotine acetylcholine receptors (nAChRs) have been exploited to facilitate BBB crossing and intracranial transport of drug delivery systems [9–12]. nAChRs are ligand-gated ion channels expressed mainly at the neuromuscular junction of central nervous system (CNS), including the brain capillary endothelial cells [13–16]. Their extensive expression in brain and susceptibility to the inhibition by peptide neurotoxins and neurotropic viral proteins enable the mediation of peptide-based transvascular delivery to brain of various therapeutic agents [11,17,18]. We previously reported the design of a 16-residue peptide, derived from the loop II region of snake neurotoxin candoxin [19,20], that bound to nAChRs with high affinity. This peptide, termed CDX, when conjugated to paclitaxel-loaded micelles, enabled drug delivery to the brain, which, in turn, inhibited tumor growth and prolonged the survival of intracranial glioblastoma-bearing mice.

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BTB resides among brain tumor cells and microvessels. Due to host site influence, BTB of malignant gliomas has less frequent trans-endothelial cell fenestrations, caveolae, vesiculo-vacuolar organelles and smaller inter-endothelial cell gaps than that of peripheral tumors microvasculature [21,22]. The pore cutoff size of different tumors implanted in cranial window is smaller than that in dorsal chamber, and the pore cutoff size of cranial U87 gliomas model is as small as 7–100 nm [23], which is narrow enough to prevent the effective transvascular passage of most nanoparticles. Integrins, which comprised a large family of heterodimeric proteins, integrate extracellular matrix components with cytoskeleton and genome [24]. Bello et al. [25] found that both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are expressed on glioma cells and neovasculature. RGD is an essential binding motif for seven out of 24 receptors of integrin (such as $\alpha_v\beta_3$) [26], making it a potent ligand to circumvent BTB in GBM [27]. RGD modified PEI gene carrier with a PEG spacer (RGD–PEG–PEI) was well-established nonviral vector for tumor-targeted gene delivery. We recently prepared RGD–PEG–PEI copolymer for the glioblastoma-targeted gene delivery. It induced the targeted gene expression of red fluorescence protein (RFP) in tumors, confirming that RGD–PEG–PEI was suitable for not only the subcutaneous, but also intracranial glioblastoma-targeted gene delivery [28,29].

Due to the triggering of caspase activation, combined use of paclitaxel, a microtubule-targeting agent, would increase the anti-tumor efficacy of TRAIL, resulting in a novel anticancer strategy [30]. It has been confirmed that adding low concentration of TRAIL to paclitaxel treated tumor cells, including glioma cells, would markedly induce cell death [31].

In the early stage of GBM, BBB integrity in intracranial glioblastoma-bearing mice is intact, but CDX–PEG–PLA–PTX micelle can circumvent BBB to facilitate brain-targeted paclitaxel delivery. With the progression of GBM, the formation of neovasculature and compromised BBB integrity result in efficient GBM-targeted gene therapy with the non-viral gene carrier RGD–PEG–PEI. It might be a promising pathway to treat GBM by combined use of brain- and tumor-targeted drug delivery systems.

2. Materials and methods

2.1. Materials

Branched PEI (Mw 25 kDa) was purchased from Aldrich (USA). Maleimide–poly(ethylene glycol)–CONHS (mal–PEG–NHS, Mw 2.0 kDa) and methoxy–poly(ethylene glycol)–maleimide (mPEG–mal, Mw 2.0 kDa) were obtained from JenKem technology Co. LTD (Beijing, China). Protected Fmoc-amino acid derivatives and Benzotriazole-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were acquired from GL Biochem Ltd (Shanghai, China). Protected Boc-amino acid derivatives were from Peptide Institute (Osaka, Japan). O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluorophosphate (HBTU) was purchased from American Bioanalytical Co. (Natick, MA). Diisopropylethylamine (DIEA) and Boc-Gly-PAM resin were supplied by Fluka (USA). Plasmid DNA encoding EGFP (pEGFP-N2) and RFP (pDsRed-N1) driven by CMV promoter were purchased from Genechem Co. (Shanghai, China). Plasmid TRAIL (pORF-hTRAIL, 4058 bp) was kindly gifted by Prof. Chen Jiang (School of pharmacy, Fudan University). Paclitaxel was kindly gifted by Prof. Hao Wang (Shanghai Institute of Pharmaceutical Industry). Alexa Flour® 488 annexin V/propidium iodiade (PI) apoptosis assay kit was from Invitrogen (USA). Other reagents were all of HPLC grade.

U87 glioblastoma cell line was obtained from Shanghai Institute of Biochemistry and Cell Biology. It was cultured in special Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco).

Male Balb/c nude mice of 4–6 weeks age were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All animal experiments were carried out in accordance with the guidelines evaluated and approved by the ethics committee of Fudan University. For the intracranial glioblastoma model, U87 cells (1×10^6 cells suspended in 5 μl PBS) were implanted into the right striatum of male Balb/c nude mice by using a stereotactic fixation device with mouse adaptor.

2.2. Preparation of CDX–PEG–PLA–PTX micelle

mPEG–PLA (methoxy–poly(ethylene glycol)–*block*-poly(lactic acid)) and CDX–PEG–PLA were synthesized as previously reported [20,32]. CDX–PEG–PLA–PTX micelle was prepared as follows: 20 mg micelle materials (containing 1 mg CDX–PEG–PLA and 19 mg mPEG–PLA) and 10 mg paclitaxel were co-dissolved in 3 ml acetonitrile, and rotary evaporated to form thin film at 37 °C. CDX–PEG–PLA–PTX micelle was obtained by hydrating the thin film with physiological saline. It was filtrated against 0.22 μm filter membrane (millipore) to remove free paclitaxel. The CDX–PEG–PLA micelle was spherical with a mean diameter of 39 nm, as analyzed by dynamic light scattering and atomic force micropic technique [20].

2.3. Preparation of RGD–PEG–PEI/pDNA nanoparticles

RGD–PEG–PEI was synthesized as previously reported [28,29]. Freshly prepared RGD–PEG–PEI solution was diluted to appropriate concentration with distilled water. Equal volume of pDNA (such as pORF-hTRAIL, pEGFP-N2 and pDsRed-N1) solution (250 μg/ml) was added to obtain N/P ratio 12:1 and immediately vortexed for 30 s at room temperature. Freshly prepared nanoparticles were used in the following experiments. RGD–PEG–PEI/pORF-hTRAIL nanoparticles, which were homogenously distributed with diameter of 73 nm, were characterized by dynamic light scattering and atomic force micropic technique [29].

2.4. In vitro gene transfer study

U87 cells were trypsinized and seeded in the 24 wells plate (Corning, NY) at a density of 2×10^4 cells per well. After 24 h incubation, RGD–PEG–PEI/pEGFP-N2 (containing 2 μg of pEGFP-N2) was added and further incubated for 12 h. The transfection agents were replaced by CDX–PEG–PLA–PTX micelle (containing 10 nM PTX, in culture medium) or fresh culture medium, and incubated for another 24 h. Cells were visualized under an IX2-RFACA fluorescent microscope (Olympus, Osaka, Japan). For quantitative analysis, cells were trypsinized and centrifuged at 1600 rpm for 10 min to obtain a cell pellet, and subsequently resuspended in PBS to analyze by a flow cytometer.

2.5. Cytotoxicity in vitro

U87 cells were seeded in the 96 wells plate (Corning, NY) at a density of 6×10^3 cells per well. After 24 h incubation, to each well RGD–PEG–PEI/pORF-hTRAIL (containing 0.5 μg pORF-hTRAIL) nanoparticles were added and incubated for another 12 h. Subsequently, it was replaced by CDX–PEG–PLA–PTX micelle containing various concentration of PTX. After further 48 h incubation, the *in vitro* cytotoxicity was determined by MTT assay [32] (PowerWave *XS*, Bio-TEK, USA) at 490 nm. To calculate the inhibitory IC50 value of PTX in combined treatment, negative control was set as RGD–PEG–PEI/pORF-hTRAIL nanoparticle treated wells. To test the time-dependent enhancement of PTX anti-glioblastoma effect by co-delivery of TRAIL gene, 200 nM PTX (in micelle) was added into the TRAIL gene-treated cells with different incubation time. The cytotoxicity of CDX–PEG–PLA–PTX was determined by the same way without RGD–PEG–PEI/pORF-hTRAIL treatment. For apoptosis analysis, cells were labeled by Annexin V/PI kit for FACS assay [33]. Analysis of cell cycle status was done via FACS as previously reported [31].