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The use of folate-PEG-grafted-hybranched-PEI nonviral vector for the inhibition of glioma growth in the rat

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ABSTRACT

Combined treatment using nonviral agent-mediated enzyme/prodrug therapy and immunotherapy had been proposed as a powerful alternative method of cancer therapy. The present study was aimed to evaluate the cytotoxicity *in vitro* and the therapeutic efficacy *in vivo* when the cytosine deaminase/5-fluorocytosine (CD/5-FC) and TNF-related apoptosis-inducing ligand (TRAIL) genes were jointly used against rat C6 glioma cells. The potency of the FA-PEG-PEI used as a nonviral vector was tested in the FR-expressed C6 glioma cells and Wistar rats. The C6 glioma cells and animal model were treated by the combined application of FA-PEG-PEI/pCD/5-FC and FA-PEG-PEI/pTRAIL. The antitumor effect was evaluated by survival assays and tumor volume. This study revealed a significant increase of cytotoxicity *in vitro* following the combined application of FA-PEG-PEI/pCD/5-FC and FA-PEG-PEI/pTRAIL treatments in C6 glioma cells. Animal studies showed a significant growth inhibition of the C6 glioma xenografts using the combined treatment. These results demonstrated that the combined treatment generated additive cytotoxic effect in C6 glioma cells in both *in vitro* and *in vivo* conditions, and indicated that such treatment method using both enzyme/prodrug therapy and TRAIL immunotherapy might be a promising therapeutic strategy in treating glioma.

1. Introduction

Glioma is the most common primary malignancy of the brain. It is known as a highly chemoresistant and radioresistant cancer with high morbidity, mortality and extremely grim prognosis. The median survival time of glioma patients is generally less than 2 years, despite multi-modality treatments with extensive surgical resection, radiotherapy, chemotherapy or immunotherapy. Recent advances in the neurosurgical technique, radiation therapy and chemotherapy have all failed to improve the survival rate of this group of patients [1]. Therefore, novel strategy is urgently needed for promoting the survival rate of the glioma patients. In recent studies, gene therapy has been proposed as one of the potential strategies which bears several advantages over conventional drug therapies [2,3]. A long-term expression of high dosage therapeutic effect could be easily achieved using gene therapy. The genetic agent could be delivered locally, and specifically to target tissues, which reduce the risk of nonspecific toxicity and ineffective dosing. In this study, the gene therapeutic strategies being investigated were based on some previously established anti-neoplastic principles, which included the use of prodrug/suicidal genes, tumor suppressor genes and immune-enhancing cytokine genes [3–6].

Among all gene therapeutic strategies now being investigated, suicide gene/prodrug system has been recognized as one of the most effective method in treating tumors. It has been revealed as a highly potent agent in treating most chemoresistant and radioresistant tumors [7–9]. One of the most widely investigated suicide gene/prodrug systems is the cytosine deaminase/5-fluorocytosine (CD/5-FC)

Abbreviations: CD, cytosine deaminase; FA, folate; FA-PEG-PEI, folate-PEG-grafted-hyperbranched-PEI; FR, folate-receptor; pDNA, plasmid DNA; PEI, poly(ethylene imine); PEG, poly(ethylene glycol); TRAIL, TNF-related apoptosis-inducing ligand; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil.

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which has been studied extensively during the last decade. Cytosine deaminase is an enzyme that could be found in bacteria and fungi. It could deaminate the nontoxic prodrug 5-fluorocytosine (5-FC) to form the highly cytotoxic 5-fluorouracil (5-FU). The latter compound then metabolite to inhibit thymidylate synthase or to act as false bases in DNA and RNA, thereby killing cells that are in the S-phase of the cell cycle [10]. By expressing the CD gene, and by administering water-soluble and low-toxicity 5-FC systemically, 5-FU is self-regenerative in tumor cells. More importantly, 5-FC prodrug is membrane permeable which has high bioavailability that could penetrate easily via the blood–brain barrier into the cerebrospinal fluid [11]. The application of 'localized' 5-FU chemotherapy might therefore avoid the toxicity associated with systemic 5-FU therapy, leading to a higher intra-tumoral concentration. In addition, the characteristic that the 5-FU could diffuse into adjacent cells via the cellular membrane would exert a powerful bystander effect [12]. This effect is essential in maximizing the therapeutic effect of gene therapy, since it is currently a challenge to direct therapeutic gene to target tumor cells [13]. As a result, regression can occur when a tumor is treated with a nontoxic level of 5-FC, even if only a small percentage of cells expresses CD.

Another effective antitumor strategy that is proposed currently is the apoptosis-inducing gene therapy. A wide variety of apoptosis-inducing molecules have been identified to combat tumor cells. Among all, the ligand-type cytokine molecules of the tumor necrosis factor (TNF) family are recognized as the best candidate. TNF-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane molecule, in which the carboxyl-terminus of the receptor-binding domain protrudes extracellularly [14]. Recombinant soluble human TRAIL has already been employed in clinical investigation as cancer therapy for it has been shown to induce apoptosis in various human cancers. It functions by triggering the apoptotic signal cascades through binding cognate receptors displayed on the cell surface. It was also noted to exhibit potent antitumor activity without induced toxicity in healthy tissue in various cancer xenograft models [15].

To date, the major challenge in gene therapy is to develop a highly effective gene delivery system with low toxicity. Nonviral vector is still an attractive option although the current agents being used displayed disadvantages, e.g. low transfection efficiency and high toxicity [16]. To overcome the problem of high cationic toxicity [i.e. polyethylene imine (PEI)] and low transfection efficiency [i.e. PEGylated PEI (PEG-PEI)], our team has linked a cell specific targeting molecule folate (FA) on polyethylene glycol (PEG). The FA-PEG was then grafted onto the hyperbranched PEI (25 kD). Folate is a common targeting ligand used for anti-cancer agents, since its target (i.e. folate receptor) is often overexpressed in tumor cells (i.e. C6 cell line) yet, rarely found in normal tissue, especially in the normal brain tissue [17]. Therefore, the FA has been used to test its enhancing effect on vector delivery in FR-enriched tumor cells such as C6 glioma cells [18]. In our pervious study, the FA-PEG-grafted-hyperbranched-PEI (FA-PEG-PEI) could effectively condense plasmid DNA (pDNA) into nanoparticles with a positive surface charge under a suitable N/P ratio of 15 [19]. In the present study, the potency of the FA-PEG-PEI which could be used as a nonviral vector was tested in the FR-expressed C6 glioma cells and Wistar rats.

2. Materials and methods

2.1. Plasmids and chemicals

Plasmids pCMVCD was kindly provided by Dr. W. Walther (Max-Delbrück-Center for Molecular Medicine, Berlin, Germany) and the pCMVTRAIL was producted by our lab. Plasmid DNAs were amplified in *Escherichia coli* and were purified according to the manufacturer's instructions (QIAGEN, CA, USA). The quantity and quality of the purified pDNA were assessed by measuring its optical density at 260 nm and 280 nm, and by electrophoresis in 1% argrose gel, respectively. The purified plasmid DNA was kept in aliquots at a concentration of 1 μg/μl. In this study, all chemicals including PEI 25,000 Da, monomethoxy PEG (mPEG-OH) 3400 Da, 5-fluorocytosine and (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were products from Sigma–Aldrich (St Louis, MO, USA). Polyplexes, i.e. the delivery agent/pDNA complexes, used throughout the present study were prepared at N/P 15 according to our previous *in vitro* results that the polyplexes received the highest transfection efficiency in C6 glioma cells whilst a low cytotoxicity at this N/P value [19].

2.2. Synthesis of delivery agents

α-Hydroxy-ε-amino-poly(ethylene glycol) (HO-PEG-NH_2) ($M_n = 3.4$ kDa, $M_w/M_n = 1.15$) was prepared according to a report by Kataoka et al. [20]. To conjugate folate to HO-PEG-NH_2, folic acid (2 mmol) was dissolved in anhydrous DMSO (20 ml). N-hydroxysuccinimide (NHS, 4 mmol) and dicyclohexylcarbodiimide (DCC, 4 mmol) were added and the mixture was stirred overnight at room temperature. The mixture was mixed with a DMSO solution of HO-PEG-NH_2, and TEA solution (pH 8.0) was added. The mixture was then filtrated, dialysized against deionized water (MWCO: 1000 Da), and lyophilized. FA-PEG-OH thus prepared was converted into FA-PEG-COOH by reaction with succinic anhydride (SA). FA-PEG-OH and SA (1:5 in molar ratio) were dissolved in 20 ml anhydrous chloroform and refluxed at 70 °C for 48 h. After chloroform was removed by distillation, polymer was re-dissolved in 20 ml deionized water and dialyzed against water for two days to remove small molecular succinic acid and succinic anhydride. Polymer solution was freeze-dried to yield pure FA-PEG-COOH. FA-PEG-PEI was synthesized and characterized as previously described [18]. In brief, FA-PEG-COOH (1 mmol) was activated with NHS (2 mmol) for 24 h in dry dichloromethane (20 ml) containing dicyclohexyl-carbodiimide (1.2 mmol) as a catalyst. The precipitated 1,3-dicyclohexylurea (DCU) was removed by filtration. The filtrate was added to diethyl ether and cooled at 4 °C for 2 h. The precipitate was collected by filtration and dried under vacuum at room temperature. Hyperbranched PEI 25 kDa and the NHS activated PEG were dissolved in PBS (pH 7.4) and magnetically stirred for 24 h at room temperature to produce FA-PEG-PEI. The mixture was purified by membrane dialyses (MWCO: 8000 Da) in distilled water for 1 day and the solution was lyophilized. Nontargeting PEG-PEI was synthesized by the same approach using the NHS/DCC chemistry. PEG grafting density: $PEG_{(mol)}/PEI_{(mol)} = 3:1$.

2.3. Cell culture

Glioma C6 cells were obtained from American Type Culture Collection (ATCC) and maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penn/strep, Invitrogen Corporation) in a humidified atmosphere of 5% CO_2 at 37 °C. When the cell confluence of 90% was reached, they were trypsinized and subcultured. All cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA, USA).

2.4. Western blotting analysis

The C6 glioma cells (1×10^5) were seeded in 24-well plate a day before transfection. They were bathed in DMEM with 10% FBS complete media and were incubated in a humidified atmosphere with 5% CO_2 at 37 °C until the cell confluence was around 70%. Four hours prior to transfection, the media was removed and replaced with fresh DMEM with 10% FBS. To test the expression of plasmids, pCMVCD and pCMVTRAIL, 1 μg of DNA was diluted in 50 μl of serum-free DMEM in an Eppendorf tube and mixed thoroughly. Based on an N/P ratio of 15, corresponding quantity of FA-PEG-PEI was added to a 50 μl serum-free DMEM in another sterilized tube and the sample was vortex mixed immediately. Both mixtures were then left to incubate at room temperature for 5 min. Samples of both tubes were then vortex mixed together and was left for incubation at room temperature for 30 min. The original cell culture media was replaced with a 100 μl complex solution whilst a 200 μl serum-free DMEM was added on top for each well. They were incubated at 37 °C for 4 h. Thereafter, the transfection medium was changed with fresh and complete DMEM culture media. At 72 h after the transfection, cells were washed twice with PBS and lysed with SDS sample buffer. Protein samples (20 μg) were separated using SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated at room temperature for 30 min in a blocking buffer (5% low fat milk, 150 mm NaCl, and 20 mm Tris–HCl, pH 7.5), and were then incubated with a sheep primary antibody (1:200 dilution) against the CD or a rabbit antibody (1:500 dilution) against the TRAIL and caspase-3 (Covance, Richmond, CA, USA). Secondary antibody, horseradish peroxidase (HRP)-conjugated anti-sheep or anti-rabbit IgG, was used to amplify the signal. The blots were developed using chemiluminescence system (New Life Science Products, Boston, MA, USA) and the results were photo-documented. The membranes were then washed again with buffer, and were rehybridized with a primary antibody for β-actin (1:500 dilutions). HRP-conjugated antibody and chemiluminescence system were used for the detection of β-actin as described earlier. The proteolysis of caspase-3 was evaluated by Western blotting by using antibodies against caspase-3 that could detect both unprocessed proenzyme and active forms after cleavage.