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Enhanced gene transfection efficiency by polyamidoamine (PAMAM) dendrimers modified with ornithine residues

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ABSTRACT

Aim of the study was to prepare and to evaluate gene transfection efficiency and cytotoxicity of the ornithine-conjugated PAMAMG4 dendrimers. Ornithine-conjugated PAMAMG4 dendrimers were prepared by Fmoc synthesis. A comparative gene transfection study between PAMAMG4 dendrimers and the surface modified dendrimers was conducted in HEK 293T, GM7373 and NCI H157G cell lines. Effect of excess of ornithine (100 μM) on transfection efficiency of the ornithine-conjugated PAMAMG4 dendrimers was investigated in separate experiment. Cytotoxicity of the dendriplexes was tested in HEK 293T cells by MTT assay. ^{1}H NMR and MALDI-TOF spectral analysis showed that about 60 molecules of ornithine (PAMAMG4-ORN60) were conjugated to a PAMAMG4 dendrimer. Preliminary studies indicated that dendriplexes at charge ratio (N/P 10) show higher transfection efficiency and presence of serum does not affect the transfection efficiency of the dendriplexes. Transfection efficiency of PAMAMG4-ORN60 dendriplexes was slightly higher in cancer cells (NCI H157G) as compared to HEK 293T cells. Transfection efficiency of the PAMAMG4-ORN60 dendrimers decreased in presence of excess of ornithine while there was no effect on the parent PAMAMG4 dendrimers. Cytotoxicity assay has shown that PAMAMG4-ORN60 dendriplexes at N/P 10 were safe at concentrations ≤50 μg/mL. It may be concluded that the ornithine-conjugated dendrimers possess the potential to be novel gene carrier.

1. Introduction

Polyamidoamine (PAMAM) dendrimers are attractive gene carriers because of their well defined structure and ease of surface modification, safety and lack of immunogenicity (Manunta et al., 2004; Lee et al., 2003; Wang et al., 2001; Chen et al., 2000; Kukowska-Latallo et al., 1996; Dufes et al., 2005; Fischer et al., 2003). Because of their net cationic charge, PAMAM dendrimers interact with phosphate groups of DNA electrostatically, condensing DNA into compact complexes called dendriplexes. The compaction of DNA by the dendrimers protects the DNA from degradation by nucleases and enhances cellular uptake of these compact particles via adsorptive endocytosis or phagocytosis (Tang and Szoka, 1997; Belinska et al., 1996; Belinska et al., 1997). Dendriplexes bind to the cell surface by electrostatic interaction with heparan sulfate proteoglycans (HSPG) and integrins ($\alpha_v\beta_3$) at the cell surface and are internalized by endocytosis (Mislick and Baldeschwieler, 1996; Mounkes et al., 1998). Once in the acidic environment of endosome, dendrimer acts as a 'proton sponge' triggering osmotic effect (Tang et al., 1996). The osmotic drag of counter ions and water leads to vesicle rupture and release of DNA complexes. Cationic dendrimers are considered advantageous over other positively charged carriers (e.g. cationic lipids) because of their extended lifetimes *in vivo* (Tang et al., 1996), whereas cationic lipid complexes are usually rapidly cleared from the circulation by the reticuloendothelial system (Kobayashi and Brechibel, 2004). Although dendrimers can carry high gene load, their major limitations for *in vivo* application are: (i) low transfection efficiency, (ii) unspecific sequestration by non-target tissues or lack of target specificity, and (iii) limited transport into the nucleus of the target cells (Zhang et al., 2005; Cassidy and Schatzlein, 2004; Schatzlein, 2003; Audouy et al., 2002; Ogris and Wagner, 2002).

Polyamines are ubiquitous in living systems and are essential for various biological processes such as cellular proliferation including tumor growth, neoplastic transformation and carcinogenesis (Pegg, 1988). Ornithine decarboxylase (ODC) is a key enzyme responsible for decarboxylating ornithine to putrescine, the rate-limiting step in polyamine biosynthesis. ODC has very rapid turnover rate (only few minutes) and is considered as a biomarker for can-

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cer (Criss, 2003). It is present both in the cytoplasm and in the nucleus (Marton and Pegg, 1995) providing the driving force for ornithine uptake by polyamine transporter system (PAT) and transportation into the nucleus as well. Many tumor types have been shown to contain elevated polyamine levels and an activated PAT for importing exogenous polyamines (Gardner et al., 2004; Cullis et al., 1999). Based on the enhanced cellular need for these amine growth factors and existence of an activated transport system for their import, we hypothesized that polyamines and their analogs can be used as biomarkers of cancer. In accordance with the hypothesis, we and other research groups have demonstrated high uptake of polyamines in various cancer cell lines, suggesting particularly ornithine and putrescine as potential biomarkers for cancer (Gardner et al., 2004; Cullis et al., 1999; Palakurthi et al., 2002; Wang et al., 2003; Bergeron et al., 1997; Kramer et al., 1993). A recent biodistribution study in AT3B-1 rat prostate tumor model by our research group has also demonstrated the potential of putrescine and ornithine as positron emission tomography (PET) imaging agents for early detection of cancer (Palakurthi et al., 2007).

Polyamines and their analogs are well known for condensing and packaging DNA into compact forms such as rods, toroids, and spheroids, which are structurally similar to phage DNA in the capsid of viruses (D'Agostino et al., 2006; Ahmed et al., 2006; Vijayanathan et al., 2001; Golan et al., 1999; Fang and Hoh, 1998; Bloomfield, 1997). Polyamines interact with DNA reversibly and the resulting DNA complexes do not withstand dilution or binding to polyanions, hence cannot be used as DNA vectors (Clamme, 2000; Remy et al., 1994). To explore the potential of polyamine-based vectors, lipopolyamines with varied unsaturation in N^4,N^9-dioctadecanoyl spermines were synthesized and their transfection efficiency and DNA condensation ability were found to be better than the Transfectam® [dioctadecylamidoglycyl spermine] formulation (Ahmed et al., 2006). Similarly, Ewert et al. (2006); have synthesized a dendritic lipid, MVLBG2 (starting from ornithine methyl ester), terminated by carboxyspermine moieties. These cationic lipids upon interaction with DNA, form hexagonally ordered cylindrical micelles embedded in a DNA honeycomb lattice. The resultant lipoplexes were found to be significantly more transfectant than commercially available, optimized DOTAP-based complexes.

Above-mentioned studies have propelled us to hypothesize that conjugation of the polyamines to PAMAM dendrimers may increase their cellular uptake by the actively growing cells such as cancer cells resulting in high transfection efficiency and cancer cell specificity of the dendriplexes. As a first step to realize the above goal, ornithine-conjugated dendrimers were prepared and their transfection efficiency was tested in various cell lines using plasmid DNA encoding for green fluorescent protein (pmaxGFP) as the reporter gene. Comparative cytotoxicity of these surface modified dendrimers and parent PAMAM dendrimers was assessed by MTT (methylthiazoletetrazolium) assay.

2. Materials and methods

2.1. Materials

PAMAM dendrimers of different generations were obtained from Dendritic Nanotechnologies Inc. (Mount pleasant, MI). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), piperidine, *N*,*N*-dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIPEA), diethyl ether, D_2O, 2,5-dihydrobenzoic acid (DHB), tetramethyl silane (TMS) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ornithine hydrochloride, paraquat and DNase I were purchased from Sigma-Aldrich (Saint-Louis, MO). Trifluoroacetic acid (TFA), ethylene diamine tetra acetic acid (EDTA), phosphate buffered saline (PBS, pH 7.4) and cell culture materials were purchased from Fisher Scientific (Chicago, IL). *N*-hydroxybenzotriazole (HOBt), and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium (HBTU) were purchased from Anaspec (San Jose, CA). Fmoc-ornithine(Boc)-OH was purchased from Novabiochem (San Diego, CA). One shot® Top10 chemically competent cells were from Invitrogen (Carlsbad, CA). Hank's balanced salt solution (HBSS) with calcium and magnesium (without phenol red) was purchased from Cellgro (Herndon, VA). Ethidium bromide (EtBr) was obtained from Fisher Bio Reagents (Fair Lawn, NJ).

2.2. Methods

2.2.1. Synthesis of ornithine-conjugated PAMAM dendrimers (PAMAM-ORN)

Ornithine-conjugated PAMAM dendrimers (generation 4) were prepared using Fmoc synthesis as reported by our research group earlier (Choi et al., 2004; Pisal et al., 2008). Briefly, to 1.0 mmol of PAMAMG4-NH_2 dendrimer in 3.0 mL of DMF, required amount of each of HOBt, HBTU, Fmoc-ornithine(Boc)-OH and DIPEA were added at a molar ratio 1:60. The reaction mixture was allowed to stir for 4 h at room temperature. The product was precipitated in about 5 mL of diethyl ether and washed with excess of diethyl ether. Fmoc groups of Fmoc-ornithine(Boc)-OH-coupled dendrimer were removed by adding 2.0 mL of 30% piperidine in DMF (v/v). After 1 h of deprotection reaction, the mixture was precipitated in diethyl ether and washed with excess of diethyl ether. Deprotection of the BOC group was achieved by 90% TFA for 1 h at room temperature and the final product was precipitated in diethyl ether and washed with excess of diethyl ether. The product was then solubilized in deionized water, dialyzed against deionized water at 4 °C overnight and then purified by Sephacryl S-300 column chromatography with acetonitrile:Tris buffer (70:30) as the elution buffer. The elution fractions corresponding to the dendrimer size were collected, dialyzed against deionized water at 4 °C, lyophilized and stored at 4 °C for further studies. The yield was always more than 95%.

2.2.2. NMR and Mass spectral analysis

^{1}H NMR spectra (chemical shift in ppm with respect to TMS set at zero) of ornithine-conjugated PAMAMG4 dendrimers (PAMAMG4-ORN60) were recorded on a Bruker AMX-400 (400 MHz) Spectrometer using D_2O as the solvent (with 0.05%, v/v TMS).

To confirm the molecular weight of surface modified dendrimers, mass spectral analysis of the dendrimers was performed on a Bruker MALDI-TOF (matrix assisted laser desorption/ionization-time of flight) using DHB as the matrix.

2.2.3. Plasmid preparation

A 3.4 kbp plasmid encoding for green fluorescent protein (pmaxGFP), with molecular weight of about 2.3 MDa (given an average of 330 Da per nucleotide, 660 Da per base pair (Felgner et al., 1997), carrying 6972 negative charges) was used as a reporter gene to monitor the results of gene transfection. DNA plasmid pmaxGFP purchased from Amaxa Inc. (Gaithersburg, MD) was transformed into One shot® Top10 cells and highly purified covalently closed circular plasmid DNA was isolated by plasmid purification mini and maxi kits from Qiagen (Valencia, CA), according to the manufacturer's instructions. Plasmid concentration and purity, $A_{260}/A_{280} > 1.9$, was assessed using Nanodrop ND-1000 Spectrophotometer (Wilmington, DE). Plasmid integrity was confirmed by 1% agarose gel electrophoresis and stored at −20 °C until further use.