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Research paper

Serum-resistant lipopolyplexes for gene delivery to liver tumour cells

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Abstract

In this study, an efficient non-viral gene transfer system has been developed by employing polyethylenimine (PEI 800, 25 and 22 kDa) and DOTAP and cholesterol (Chol) as lipids (lipopolyplex), at three different lipid/DNA molar ratios (2/1, 5/1 and 17/1) by using five different protocols of formulation. Condensation assays revealed that PEI of 800, 25 and 22 kDa were very effective in condensing plasmid DNA, leading to a complete condensation at N/P ratios above 4. Addition of DOTAP/Chol liposomes did not further condense DNA. Increasing the molar ratio lipid/DNA in the complex resulted in higher positive values of the ζ-potential, while the particle size increased in some protocols, but not in others. High molecular weight PEI (800 kDa) used in the formulation of lipopolyplexes lead to a bigger particle size, compared to that obtained with smaller PEI species, whether branched (25 kDa) or linear (22 kDa). These vectors were also highly effective in protecting DNA from attack by DNAse I. Transfection activity was maximal by using protocols 3 and 4 and a lipid/DNA molar ratio of 17/1. These complexes showed high efficiency in gene delivery of DNA to liver cancer cells, even in the presence of high concentration of serum (60% FBS). On the other hand, complexes formed with linear PEI (22 kDa) were more effective than lipopolyplexes containing branched PEI (800 or 25 kDa). The complexes resulted to be much more efficient than conventional lipoplexes (cationic lipid and DNA) and polyplexes (cationic polymer and DNA). The same behaviour was observed for complexes prepared in the presence of the therapeutic gene pCMVIL-12. Toxicity assays revealed a viability higher than 80% in all cases, independently of the protocol, molar ratio (lipid/DNA), molecular weight and type of PEI.

Keywords: Gene delivery; Cationic liposomes; Polyethylenimine; Lipopolyplexes; Non-viral vectors; Cancer gene therapy

1. Introduction

Gene therapy focuses on the therapeutic use of genes delivered to cells and promises considerable advances in the treatment of several important diseases. Although remarkable progress has been made in identifying target structures for cancer gene therapy, actual therapy is mainly hampered by the lack of a safe and efficient delivery system. Thus, one of the primary objectives of gene therapy is the development of efficient, non-toxic gene carriers that can effectively deliver foreign genetic material into specific cell types, including cancerous cells.

The two main gene carrier systems that have been utilized in gene therapy are viral vectors and non-viral delivery systems. Viral vectors, including retroviruses, adenoviruses and adeno-associated viruses, have a high efficiency of gene delivery. Due to serious safety risks with viral vectors that have become apparent in the last few years, however, their utility is being re-appraised. Furthermore, the addition of targeting ligands on the surface of viral vectors to transfect specific cell types is problematic. Because of these concerns, non-viral vectors are emerging as a viable alternative. Non-viral systems show significantly lower safety risks, they are capable of carrying large molecules and can be produced in large quantities easily and inexpensively. The major disadvantage of these

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non-viral-vectors is their low transfection efficiency, especially in the presence of serum in vivo.

Different strategies including particle bombardment [1], ultrasound transfection [2] or the application of naked DNA [3] have been used as gene delivery systems. Their applicability, however, is restricted to specific circumstances. Cationic polymers and lipids are by far the most widely used vectors in non-viral gene and oligonucleotide delivery. Some liposome formulations are not satisfactory due to their low transfection efficiency and cytotoxicity [4,5]. Polycationic polymers are able to compact DNA, which is an advantage in gene transfer [6,7]). Polyethylenimine (PEI) has been used successfully for in vitro and in vivo gene delivery [8,9]. It plays several roles in the process of transfection, such as condensing and protecting DNA, binding to the cell surface, triggering endocytosis and releasing DNA/lipid complexes from endosomes due to the ''proton-sponge'' effect [10]. It has the ability to enter the nucleus [11] and to accelerate gene entry into the nucleus from the cytosol [12]. Although cationic liposomes can deliver DNA into the cytosol through the endosomal pathway, the entry of the DNA into the nucleus is very inefficient [13,14]. Lipopolyplexes (i.e. a ternary complex of cationic liposomes, cationic polymer and DNA) represent a second generation of non-viral gene delivery vectors that can improve gene transfer compared to the first generation cationic liposome–DNA complexes (lipoplexes) [6,15–20].

Because of our interest in gene therapy of hepatocellular carcinoma, we have examined the ability of various lipopolyplexes to deliver genes into liver cancer cells. We evaluated different parameters such as the protocol of preparation, the lipid/DNA molar ratio, and the molecular weight and type of PEI, to optimize the formulation to achieve high transfection activity. Our hypothesis was that the association of PEI with cationic liposomes (lipopolyplexes) would increase luciferase expression compared to lipoplexes (cationic lipid and DNA) and polyplexes (cationic polymer and DNA) alone.

2. Materials and methods

2.1. Materials

The cationic lipid 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Polyethylenimine 800 (MW 800 kDa, branched) was purchased from Fluka (Steinheim, Germany), polyethylenimine 25 (MW 25 kDa, branched) from Aldrich (Madrid, Spain) and linear PEI (MW 22 kDa, ExGen® 500) from Quimigranel (Madrid, Spain). The plasmids, pCMVLuc (VR-1216) (6934 bp) (Clontech, Palo Alto, CA, USA) and pCMV100-IL-12 (5500 bps) (kindly provided by Dr. Chen Qian, University of Navarra) encoding luciferase and interleukin-12 (IL-12), respectively, were used for carrying out the transfection experiments. The following materials were used for DNAse I protection assays: agarose D-1 (Pronadisa, Madrid, Spain), Tris-boric acid–EDTA Buffer (10× TBE Buffer) (Invitrogen, Barcelona, Spain), DNAse I and ethidium bromide (Gibco BRL, Barcelona, Spain). To release DNA from the complexes sodium dodecyl sulphate (SDS) and NaCl were used (Roig Farma, Barcelona, Spain); heparin sodium salt and ethylenediaminetetraacetic acid (EDTA) were supplied by Sigma (Madrid, Spain). Alamar blue dye was purchased from Accumed International Companies (Westlake, OH, USA).

2.2. Cell culture

HepG2 human hepatoblastoma cells (American Type Culture Collection, Rockville, MD, USA) were maintained at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium-high glucose (DMEM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (4 mM) (Gibco BRL Life Technologies). Cells were passaged 1:10 by trypsinization once a week.

2.3. Preparation of lipopolyplexes

Lipopolyplexes were prepared with plasmid DNA and B-PEI (branched, 800 or 25 kDa) or L-PEI (linear, 22 kDa) at a N/P ratio of 4. The N/P ratio of the nitrogen atoms of PEI to DNA phosphates describes the amount of polymer used for polyplex formation. Different amounts of lipids were added in order to prepare complexes at molar ratios of total lipid/DNA of 2/1, 5/1 and 17/1. The final DNA concentration in the lipopolyplexes was 25 μg/ml in 3 ml of total volume.

Lipopolyplexes were formulated by five different strategies: *Strategy 1* was to dry a chloroform solution of the lipids DOTAP/Chol (1:0.9 molar ratio) by rotary evaporation and then to hydrate the film with the polyplexes (PEI/DNA) at a N/P ratio of 4. *Strategies 2 and 3* were performed by drying a chloroform solution of the lipids (DOTAP/Chol) and PEI by rotary evaporation. In strategy 2 the film was hydrated with water, followed by the addition of the plasmid. In strategy 3, the mixture of lipids and PEI were directly hydrated with the plasmid. *Strategies 4 and 5* were to formulate the polyplexes PEI/DNA at a N/P ratio of 4 and, after a 15-min incubation, to add different amounts of preformed cationic liposomes, to obtain complexes at different lipid/DNA molar ratios (2/1, 5/1 and 17/1). Both strategies are similar, differing only in the order of addition of plasmid to obtain the polyplexes: PEI added to DNA (strategy 4) or DNA added to PEI (strategy 5). All the complexes were extruded through polycarbonate membranes with 100 nm pore diameter using a Liposofast device (Avestin, Toronto, ON, Canada) to obtain a uniform size distribution.

2.4. DNA/PEI condensation assays

The binding of PEI to DNA was examined using a quenching method based on ethidium bromide, whose