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Effectiveness, against tuberculosis, of pseudo-ternary complexes: Peptide-DNA-cationic liposome

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ABSTRACT

We report the effects of a synthetic peptide designed to act as a nuclear localization signal on the treatment of tuberculosis. The peptide contains 21 amino acid residues with the following specific domains: nuclear localization signal from SV 40T, cationic shuttle sequence, and cysteamide group at the C-terminus. The peptide was complexed with the plasmid DNAhsp65 and incorporated into cationic liposomes, forming a pseudo-ternary complex. The same cationic liposomes, composed of egg chicken L-α-phosphatidylcholine, 1,2-dioleoyl-3-trimethylammonium-propane, and 1,2-dioleoyl-3-trimethylammonium-propane (2:1:1 M), were previously evaluated as a gene carrier for tuberculosis immunization protocols with DNAhsp65. The pseudo-ternary complex presented a controlled size (250 nm), spherical-like shape, and various lamellae in liposomes as evaluated by transmission electron microscopy. An assay of fluorescence probe accessibility confirmed insertion of the peptide/DNA into the liposome structure. Peptide addition conferred no cytotoxicity *in vitro*, and similar therapeutic effects against tuberculosis were seen with four times less DNA compared with naked DNA treatment. Taken together, the results indicate that the pseudo-ternary complex is a promising gene vaccine for tuberculosis treatment. This work contributes to the development of multifunctional nanostructures in the search for strategies for *in vivo* DNA delivery.

1. Introduction

DNA vaccines have become a promising alternative for the treatment and prevention of different infectious diseases [1–3], such as tuberculosis [4]. Typically, this kind of vaccine consists of a modified bacterial plasmid DNA coupled to a gene sequence that encodes a desired protein. Regarding the several methods of *in vitro* or *in vivo* DNA administration, the DNA will reach the cell nucleus, be transcribed into mRNA, and translated to the corresponding protein. If this DNA is captured by specialized antigen presenting cells (APCs), the endogenously produced protein will be processed to small peptides and presented on the cell surface, stimulating the T cell adaptive immune response. Also, the entire protein could be secreted from the cell and recognized by B cells, stimulating specific antibody production [5]. This versatile and broad range of immune response activation makes plasmid DNA a very useful tool.

However, naked DNA is rapidly degraded *in vivo* by extracellular deoxyribonucleases and exhibits poor cellular uptake. An alternative to overcoming this difficulty is the development of safe and efficient gene carriers [6]. In this context, cationic liposomes can be used as non-viral carriers, protecting and directing gene material to the cells [7,8]. The mechanism of DNA transfection by cationic liposomes has been studied by many authors, and these gene carriers are well known to deliver DNA efficiently into the cytosol [9–12]. After DNA release into the cytosol, transfection success still depends on DNA trafficking toward the nucleus, and its transport across the nuclear envelope still remains one of the most important barriers [13].

During cell division, eukaryotic cells have a disordered nuclear envelope, facilitating DNA entrance. However, if the cell is not dividing, the nuclear envelope is an important barrier for macromolecules, such as plasmid DNA [13,14]. The nuclear envelope consists of a highly ordered membrane containing pores that allow the

Abbreviations: NLS, nuclear localization signals; EPC, L-α-phosphatidylcholine (egg, chicken); DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DMB-Chol, cholesteryl-3 β-carboxybutylene-N-dimethylamine; LPS, lipopolysaccharide; ERK, extracellular signal-regulated kinases; IL, interleukin; MTT, 3-(4,5-diethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide.

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passive diffusion of small molecules [15,16]. Larger molecules can be transported through the nuclear pore by specialized cargo proteins, and one well-known transport mechanism is through nuclear localization signals (NLS). NLS basically consist of a short peptide sequence within proteins that allows association of the molecule with specific cargo proteins that provide nuclear transport [17]. In this context, NLS represents an additional strategy and can be incorporated into non-viral gene carriers, such as liposomes, focusing the improvement of plasmid DNA delivery inside the nucleus to overcome nuclear macromolecule exclusion. Different research groups have designed cationic synthetic peptides containing NLS, increasing *in vitro* and *in vivo* transfection rates [18]. The overall cationic nature of peptides allows DNA condensation. Byrnes and colleagues covalently coupled the M9 sequence to the cationic peptide (scrambled sequence of the cationic SV40 T antigen—KCRGKVPGKYGKG) [19]. The binary DNA/peptide complex was associated to lipofectamine (cationic lipid carrier) and *in vitro* transfection increased 20-fold. Recent studies demonstrated that lipofectamine (commercial cationic lipid vector) associated with DNA/peptide containing the minimal SV40 T-antigen NLS (EGPKKKRKVG) and a scrambled version of SV40 (EKRGKVKPKG) successfully transfected cells. Relative to lipid alone, peptide–lipoplexes enhanced *in vitro* transfection by up to 4.6-fold. The presence of the peptide in the lipoplex increased the internalization efficiency up to 4.5-fold, decreased the percentage of lysosomal DNA by 2.1-fold, and increased the efficiency of nuclear accumulation by 3.0-fold [20]. In an *in vivo* model, Schirmbeck and colleagues demonstrated that mice immunized with DNA encoding the hepatitis B surface antigen (HBsAg) coupled to NLS exhibited a 10–15-fold increase in HBsAg-specific antibody production compared with uncoupled vector [21]. Moreover, in *Leishmania major* challenged mice, immunization with linear DNA coupled with NLS offered greater protection than a prime-boost administration protocol [22].

We developed a DNA vaccine encoding the 65-kDa heat shock protein gene from *Mycobacterium leprae* (DNAhsp65), which exhibits prophylactic and therapeutic effects on mice with tuberculosis (TB) [23–26]. This vaccine elicits robust CD4 and CD8 memory T cells, with CD8 being the prominent cell population, resulting in cytotoxic activity and IFN (interferon)-γ cytokine production [27,28], fundamental elements for killing mycobacteria. Furthermore, DNAhsp65 immunotherapy suppressed TH2 cytokine levels (which oppose IFN-γ effects) and controlled the intensity of local inflammation [29,30]. In this sense, the fine-tuning of the host immune system induced by DNAhsp65 administration is the key for optimal results in combating the *Mycobacterium tuberculosis* pathogen.

Although these results were encouraging, the amount of plasmid DNA necessary for this goal was high. Thus, our research group developed a cationic liposome containing DNAhsp65 containing the lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and L-α-phosphatidylcholine (EPC) to sustain protection against TB and decrease the administered dose of DNA. The lipid composition was the same as originally developed by Perrie and colleagues [31]; therefore, the DNA content was increased in the lipid structures and then complexed with preformed dehydrated–hydrated vesicles (DRV). Preclinical results demonstrated the prophylactic potential of liposome genetic vaccine, with low cytotoxicity added to the advantage of being a single-dose intranasal vaccine with advantageous results for DNA complexed with DRV liposomes instead of conventional encapsulation [32,33]. Thus, in the present study, our aim was to design a non-viral gene carrier with multifunctional domains that combines a new synthetic peptide containing a NLS with DNAhsp65/cationic liposome (previously developed [32,33]) useful for tuberculosis treatment. The pseudo-ternary (NLS/DNA/cationic liposome) complex was characterized according to its physical–chemical properties, including average diameter, zeta potential, gel retardation assay, and accessibility to DNA fluorescent probe, and it was also evaluated for cytotoxicity *in vitro* and therapeutic effect *in vivo*.

2. Materials and methods

2.1. Materials

Plasmid pVAX1 encoding no gene (DNAmock) or pVAX-hsp65 encoding a 65 KDa heat shock protein from *M. leprae* (DNAhsp65) was designed for a tuberculosis gene vaccine and supplied by the Center for Tuberculosis Research, Medical School of São Paulo University at Ribeirão Preto [4]. EPC, DOPE, and DOTAP chloride salt were purchased from Avanti Lipids and used without further purification.

2.2. Peptide synthesis and purification

The KCRGKVPGKYGKGPKKKRKVC-amide, the NLS, was synthesized as described previously [34]. The crude peptide was submitted to O_2 oxidation in aqueous solution at neutral pH for 20 h. Peptide purification was carried out on a Waters 510 HPLC instrument using a Vydac C18 preparative column (22-mm internal diameter, 250-mm length, 70-Å pore size, 10-μm particle size). The peptide was eluted with a linear gradient using H_2O containing 0.1% TFA (solvent A) and 60% acetonitrile in H_2O containing 0.1% TFA (solvent B). A linear gradient (25–55% B) over 90 min with a flow rate of 10 mL/min and UV detection at 220 nm was used. The fractions were screened under isocratic conditions in a Chromolit C18 analytical column. Pure fractions of the S-S cyclized peptide were pooled, lyophilized, and characterized for homogeneity by analytical HPLC (Waters Associates, Milford, MA, USA), mass spectrometry on RP-HPLC/MS (Micromass, Manchester, UK), and amino acid analysis (Biochrom 20 Plus, Amersham Biosciences, Uppsala, Sweden).

The 21 amino acid residues of the synthesized peptide had the following specific domains and characteristics: (i) NLS from SV 40 T (simian virus), 7 residues (PKKKRKV) [35], (ii) cationic amino acid sequence derived from SV 40 T, shuttle sequence with 13 residues (KCRGKVPGKYGKG) [19,36] with the purpose of improving electrostatic interactions with DNA, (iii) a cysteamide group at the C-terminus [37,38], which was also acetylated at the N-terminus to increase transfection efficiency, and (iv) because this peptide contains two cysteine residues, we promoted cyclization to avoid any uncontrolled disulfide bridge formation during complexation with DNA.

2.3. Preparation of binary (NLS/DNA) and pseudo-ternary (NLS/DNA/cationic liposomes) complexes

The binary NLS/DNA complex was formed by adding the appropriate amount of peptide to DNA aqueous solution while vortexing for 40 s. The binary complex was incubated at 4 °C for 10 min before use. The peptide/DNA proportion was established in terms of molar charge ratio (+/−) between the peptide positive charges (NH_4^+) and DNA negative molar charges (PO_4^{3-} groups) ($R_{NLS+/DNA-}$). This balance was based on the physiological pH. In this special case, 1 mol of peptide (Mw = 3484.97) corresponds to 10 mols of positive charge and 1 μg of DNA corresponds to 3 nmols of negative charge [39].

The pseudo-ternary complex was obtained by adding the binary complex to the cationic liposomes while vortexing for 40 s. This complex was kept at room temperature for at least 30 min before physico-chemical and *in vivo* characterization. This formulation