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Novel cationic liposomes provide highly efficient delivery of DNA and RNA into dendritic cell progenitors and their immature offsets

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ABSTRACT

Here we report on the application of cationic liposomes formed by new cationic lipids and the lipid-helper DOPE (dioleoylphosphatidylethanolamine) for the transfection of plasmid DNA and mRNA into dendritic cells (DCs) progenitors and immature DCs of bone-marrow origin *in vitro* and the use of these DCs to induce the suppression of B16 melanoma metastases *in vivo*. The cationic lipids contain one (*X*2, S1, S2 and S3) or two (2X3) cholesterol residues or long-chain hydrocarbon substituent (2D3) linked with spermine. Data show that liposomes 2X3-DOPE, 2D3-DOPE, *X*2-DOPE and S2-DOPE display high transfection efficiency in respect to DNA (30–47% of DC progenitors and up to 57% of immature DC were transfected) and RNA (up to 57% of cells were transfected). The studied lipids exhibited an efficiency of DNA and RNA delivery in DCs several times higher in comparison with Lipofectamine 2000. Observed *ex vivo* the higher transfection efficiencies of DCs with mRNAs encoding of a set of tumor-associated antigens provided by cationic liposomes 2X3-DOPE and 2X2-DOPE corresponded to a 3–5 fold suppression of metastasis number in a model of murine B16 melanoma *in vivo*. The injection into mice of these pulsed DCs resulted in a slight pro-inflammatory response which was balanced by the positive effect of the antitumor cytokine production induced by the DCs. The obtained data show that the novel spermine-based polycationic lipids can be applied in the preparation of antitumor DC-based vaccine.

1. Introduction

Dendritic cells (DCs) are specialized antigen-presenting cells that capture, process and present tumor-associated antigens (TAA) to T-cells, subsequently triggering antigen-specific antitumor immune responses. Immunization of patients with DCs pulsed with TAA is a prospective approach of antitumor therapy, successfully used in clinical trials [1]. Recently, the first US Food and Drug Administration approval in history was granted, for a therapeutic DC-based cancer vaccine. This was the result of a randomized clinical trial of Sipuleucel-T (Provenge; Dendreon, Inc.), an autologous dendritic cell based vaccine loaded with a prostatic acid phosphatase (PAP)-granulocyte-macrophage colony stimulating factor (GM-CSF) fusion protein used to treat men with advanced castrate resistant prostate cancer [1].

DCs can be loaded with tumor antigens in the form of peptides, proteins, lysates of tumor cells, mRNAs and plasmid DNAs (pDNAs) encoding TAA. DCs are able to uptake antigens via different ways depending on their mature/immature status: macropinocytosis (capture of soluble antigens) [2], phagocytosis (capture of viruses, bacteria, necrotic and apoptotic cells) [3], and receptor-mediated endocytosis realizing through lectin, complement CR3 and Toll-like receptors [4]. The efficiency of the immune response triggered by DCs directly depends on the form of TAAs and efficiency of antigen acquisition by DCs. However, many of the molecular changes that allow a tumor to grow and infiltrate its environment (chronic inflammation, infiltration of tumor with myeloid-derived suppressor cells and tumor-associated macrophages [5,6]) also create an immunosuppressive milieu that counteracts DC-mediated tumor rejection in particular through the diminishing or complete lockout of the uptake of TAAs by DCs [7] In this regard and by virtue of the fact that DCs are by themselves difficult targets for TAA delivery due to the alterations in their ability to capture antigen at maturation, the problem of DC loading with TAA is very apparent.

In contemporary times various approaches for antigen delivery into DCs are used. Among these approaches are viral vectors (most commonly adenoviral [8], retroviral [9–11] and lentiviral vectors [12,13]) and non-viral methods [14] including electroporation [15], lipofection [16,17] and polycationic complexes [18]. Electroporation is the most effective method among non-viral approaches but with relatively high cell mortality (29%) [19]. It has been demonstrated that DCs transduced with viral vectors expressing TAA stimulate strong antitumor responses *in vitro* [20,21], induce protective

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immunity *in vivo* in tumor models [22–27] yet provide little clinical benefits in Phase I clinical trials [28–30]. The main disadvantages of viral vectors: possible mutagenesis and carcinogenesis as a result of viral genome integration into the DNA of host cells, as well as the immunogenicity of viral proteins have yet to be resolved.

Non-viral chemical vectors, such as liposomes and polycationic complexes, have been under intense development during recent years because of their low immunogenicity and the simplicity of their synthesis and modification [31]. It has been demonstrated that cationic polymers efficiently deliver antigens into DCs and can elicit effective immune responses [32,33].

Cationic liposomes have displayed a great potential for delivering antigens into DCs [17,34]. To improve the uptake of nucleic acids by DCs using receptor-mediated routes mannosylated liposomes [35–37], liposomes containing trimethyl ammonium propane [38], and phosphatidylserine [39] targeted to mannose receptor (MR), negatively charged surface proteins and PS receptor of DCs were designed [40] However despite the large number of designed systems for delivery of nucleic acids into DCs the problem of highly-efficient antigen delivery providing the efficient antigen expression and presentation is still unsolved.

Herein we studied the ability of novel cationic liposomes to mediate the delivery of plasmid DNA and RNA into DC progenitors and immature DCs of bone-marrow origin. The polycationic lipids under study contain one (*X*2, S1, S2 and S3) or two (2X3) cholesterol residues or long-chain hydrocarbon substituent (2D3) linked with spermine. We found several liposomal formulations which provide efficient cellular delivery and transgene expression both in DC progenitors and their immature offsets. Studied liposomal formulations loaded with RNA encoding TAA induce DC-mediated antitumor response *in vivo* and 3–5-fold decrease in the number of lung metastases, as compared to control. These pulsed DCs caused in mice slight pro-inflammatory response which was balanced by a positive effect of the DC-induced antitumor cytokine response. The obtained data clearly show that novel cationic liposomes facilitate the efficient transfection of DCs with nucleic acids (DNA or RNA) encoding TAAs.

2. Materials and methods

2.1. Polycationic lipids and liposomes

Polycationic lipids 2X3 and *X*2 were synthesized as described previously [41]. Lipid 2D3 was synthesized starting from 1,2-di-*O*-tetradecyl-*rac*-glycerol as reported for lipid 2X3. Synthesis of lipids S1, S2, S2 will be published elsewhere; the physical–chemical characteristics of the lipids synthesized are in the Supplementary materials.

For preparation the liposomes, polycationic lipids were mixed with the DOPE (Avanti Polar Lipids, USA) solution in chloroform in 1:1 ratio, and the solvent was carefully removed under vacuum in a rotary evaporator. The obtained lipid film was dried under a vacuum (0.01 Torr) for 2 h, and was hydrated using deionized water (MilliQ) at 4 °C overnight. The lipid suspensions were sonicated for 15 min at 60–65 °C for 2X3 and 2D3 or 10 min at 50 °C for other lipids in a bath type sonicator (Bandelin Sonorex Digitec DT 52H, Germany). The resulting concentration of polycationic lipid in the liposomal formulation was 1 mM.

2.2. Cell lines

B16, the C57Bl/6J-derived melanoma cells, was purchased from Institute of Cytology (Russian Academy of Sciences, St. Petersburg, Russia). The genetically modified BHK IR780 cell line constantly expressing EGFP protein was derived from BHK (hamster kidney) cells and kindly provided by Prof. V. Prasolov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia). BHK IR780 cells were maintained in the DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin in a humidified atmosphere containing 5% CO_2 at 37 °C and was regularly passaged to keep the exponential growth.

2.3. Mice

Male 10–12 week-old C57Bl/6J (hereinafter, C57Bl) mice obtained from the animal breeding facility within the Institute of Cytology and Genetics SB RAS (Novosibirsk, Russia) were used in this study. All animal procedures were carried out in accordance with the approved protocol and recommendations for proper use and care of laboratory animals [European Communities Council Directive 86/609/CEE].

2.4. Generation of DC progenitors and their immature offsets

DC progenitors were obtained from the bone marrow of C57Bl mice by density gradient centrifugation through HISTOPAQUE-1083 medium (Sigma, USA). The CD11c+ DC progenitors were isolated using Dynabeads DC Enrichment Kit (Invitrogen, USA) according to the manufacturer's recommendations. Immature DCs were generated by culturing of CD11c+ DC progenitors in IMDM supplemented with 10% of FBS and 1% antibiotic-antimicotic solution (10 mg/ml streptomycin, 10,000 IU/ml penicillin, and 25 μg/ml amphotericin; ICN, Germany) in the presence of GM-CSF (20 ng/ml) and IL-4 (50 ng/ml) at 37 °C in a humidified atmosphere containing 5% CO_2 for 6 days.

2.5. Preparation of the complexes of cationic liposomes and nucleic acids

For the plasmid DNA transfection, pEGFP-C2 plasmid (Clontech, Heidelberg, Germany) was used. The isolation of total RNA from BHK-IR780 cells (hereinafter RNA-EGFP) or B-16 murine melanoma cells (hereinafter RNA-B16) was performed using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol.

Prior to their use, the complexes of the cationic liposomes and nucleic acids were formed in a serum-free Opti-MEM medium (Invitrogen, USA) via vigorous mixing of liposome solution and pEGFP-C2 DNA or RNA-EGFP or RNA-B16 taken at an appropriate concentration; the resulting mixtures were incubated for 20 min at room temperature. DNA(RNA)/liposome complexes were formed at P/N (phosphate to nitrogen) ratios: 1/4, 1/6, 1/8 and 1/10 for DNA transfection or 1/1 and 1/2 for RNA transfection.

2.6. Cellular delivery of pEGFP-C2 DNA and RNA-EGFP or RNA-B16

The transfection efficiency (TE) for the nucleic acids complexed with liposomes was evaluated in experiments with pEGFP-C2 DNA and RNA-EGFP.

For transfection experiments, cells were seeded into 24-well plate (DC progenitors, 5×10^5 cells/well, immature DC, 4×10^5 cells/well) in the DMEM 30 min before transfection. To formulate pDNA-containing lipoplexes pEGFP-C2 DNA (0.5 μg per well) was mixed with liposomes at concentrations corresponding to P/N ratios: 1/4, 1/6, 1/8, and 1/10. To form RNA-containing lipoplexes RNA-EGFP or RNA-B16 (5 μg per well) was mixed with liposomes at liposome concentrations corresponding to P/N ratios of 1/1 and 1/2. The lipoplexes were formed in 100 μL of serum-free Opti-MEM medium as described above. Cells were incubated with these lipoplexes for 4 h in a humidified atmosphere with 5% CO_2. FBS was then added to the medium up to concentration 10% and the cells were incubated for 44 h in the atmosphere with 5% CO_2. Transfection of DNA or RNA with commercial available Lipofectamine 2000 (Invitrogen, USA) was performed according to the manufacturer's protocols. The efficiency of transfection was evaluated by flow cytometry.