Coated cationic liposome-based lipid carriers for antisense therapy

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INTRODUCTION

Gene therapy is a promissible strategy for the prevention and treatment of many diseases. Despite their high transfection efficiency, viral vectors have serious drawbacks since immunogenicity and insertional mutation cannot be excluded (Nienhuis, 2008). Non-viral lipid carriers were developed as an alternative for a viral type gene vectors were in recent years. They were mostly cationic lipid–based gene delivery systems which were developed as a tool for gene delivery (Reimer et al. 1995). Although they proved efficient in vitro still many problems remain to be solved before using them as drug carriers. Gene transfer vector should be safe, stable, cost-effective to manufacture in clinically relevant quantities, and capable of efficient and tissue-specific delivery. Properly designed lipid-based carriers are able to fulfill all these conditions. Although there are a number of available promising results of *in vitro* tests, many obstacles must be overcome in order to improve selective and effective gene delivery into target cells and tissues. These include genetic drug protection against enzymatic degradation, biocompatibility of lipid carriers, specific delivery of genetic drugs-lipid carrier complex to target cells and tissues, and satisfactory pharmacokinetics *in vivo* (for review see Wyrozumska et al. 2006). The most promising results were obtained in transfection experiments using various cationic lipid supramolecular aggregates. These aggregates containing various cationic lipids exhibit a diversity of transfection efficacy, immunogenicity, toxicity and stability in the presence of serum. The therapeutic agents in gene therapy can be plasmid DNA, antisense oligonucleotides or siRNA which are not able to diffuse far from the injection site or to cross barriers such as endothelium or the blood-brain barrier, as they possess a high positive charge. Moreover, the net positive charge of these particles promotes opsonization and clearance from the circulation by the macrophage systems.
In this report we present a lipid-based construction which uses a concept of CCL (coated cationic liposomes) of Stuart et al (2004). Lipid composition used in the construction of such liposomes allowed us to obtain a nucleic acid carrier which is characterised by high transfection efficacy towards cells of lymphoid and myeloid origin, low toxicity when not containing antisense ODN's, lack of haemolytic activity, stability in the presence of serum, good size stability during storage.

MATERIALS AND METHODS

Lipids: DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), DOPE (dioleoyl phosphatidyl-ethanolamine), PC (phosphatidylcholine), DSPE-PEG (1,2-distearoyl-*sn*-glycero-3-phospho-ethanolamine-N-[amino(polyethylene glycol)2000](ammonium salt),DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) and DC-CHOL (3b-N-[dimethylaminoethane]carbamoyl)-cholesterol were from Nothern Lipids or Avanti asODN's were synthesised by Oligo.pl, plasmids coding for GFP or dsRED were from Invitrogen.
Lipid carrier preparation was based on the idea of others (Stuart et al., 2000, 2004) where ODN's or plasmid DNA is extracted into organic (chloroform phase) using our own lipid composition: pDNA or ODN's aqueous solution (150 mg in 150 ml) was mixed with a chloroform solution of DOTAP (3

mg in 150 ml). Next step was titration with methanol until single phase arises. Subsequent addition of chloroform (600 ml) and water (600 ml) restores two phases. After short centrifugation (7 min, 800 x g) the upper phase was discarded. To the lower (chloroform phase) containing approximately 90% of DNA or ODN's (Reimer et al., 1995) a chloroform solution of a mixture of coating lipids (HPC, 4.3 mg, DC-CHOL, 0.6 mg, DOPE, 1.5 mg, DSPE-PEG, 1.7 mg) and 250 ml of PBS (5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 2 mM KCl, 150 mM NaCl, pH 7.4) were added. Resulting suspension was sonicated (1 min, 4W in a Microson sonifier) and organic solvents are removed by evaporation in a rotary evaporator. Obtained liposome suspension after dilution with PBS to the final volume of 1 ml was extruded first through 400-, next 200- and finally through 100 nm Nucleopore filters (Whatman). Liposome diameter and zeta-potential were measured using a Malvern ZetaSizer 5000. The encapsulated nucleic acid content in such a carrier was evaluated by spectrophotometric assay (absorbance measurements at 260 nm) after extraction of lipids from the liposome suspension with a chloroform:methanol mixture (3:1).
For transfection assays, Jurkat T, K562 or HL-60 cells were seeded in 24-well plates (0.2-1.0 x 10^6 cells per well in 0.5 ml of PBS, or RPMI 1640 in the presence or absence of serum). Following addition of the plasmid DNA-containing liposomes cells were incubated for 3-8 hours and then medium was replaced by fresh PBS or RPMI medium with or without 10% FBS. After 24-48 hours incubattion they were prepared for fluorescence microscope observations by fixation in 4% paraformaldehyde in PBS buffer. Transfection efficiency was evaluated by comparing the number of the fluorescent cells to the number of all cells in the microscope field. Microscope fields containing at least 75-100 cells were evaluated. The toxicity assays were based on 2.5 % Trypan Blue exclusion test. Haemolytic activity of the liposomes was assessed by 30 min incubation at 37°C of freshly prepared human erythrocytes (0.4%) in 0.9% NaCl in 10 mM Tris-HCl pH 7.4. The percent of haemolysis was determined by assessing haemoglobin content spectrophotometrically at 570 nm in the supernatant after centrifugation at 650 x g for 10 min.
Reverse transcriptase PCR and Western Blot analyses after the treatment of leukaemic cells with asODN directed against bcl-2 gene was performed by using primers directed against bcl-2 and b-actin. RNA isolation and RT PCR reaction was.carried out by using RNeasy(Qiagen) and Robust T 1 RT-PCR (Finzyme) kits respectively. RT-PCR products were detected after agarose gel electrophoresis. Western blotting was performed on CCL-asODN's (anti bcl-2 gene)-treated or untreated cell extracts ("RIPA" buffer) by using rabbit antiBcl-2 (human) antibodies (Santa Cruz) at 1:300 dilution followed by horse radish peroxidase conjugated goat antirabbit antibodies at 1:2000 dilution(Santa Cruz). The antigen-antibody complex was visualised by chemiluminescent reaction and scanned in a BioRad VersaDoc apparatus.

RESULTS AND DISCUSSION

Our attempt to construct stable, coated cationic liposome preparation containing small amount of DSPE-PEG (see Materials and Methods) resulted in reasonably stable diameter upon storage at 4°C for 12 months and in a freeze-dried form in the presence of cryoprotectant at -20°C for 3 months and also maintained the high level of encapsulated plasmid DNA it proved stable when incubated for 3 hours in the presence of 50% human serum.and permitted chemical modification of liposomes in order to facilitate the covalent attachment of antibodies directed against specific marker of the specific cell line. Below the parameters and functional properties of obtained liposome preparation are described.

The diameter of obtained liposomes CCL-PEG was 105±15 nm and their z-potential was -8 to -10 mV. The amount of encapsulated pDNA or as ODN's was in range of 120 mg/ml. (about 90-94% of DNA used for liposome preparation was entrapped inside the carrier). When stored as a supension for up to 12 months their diameter and its distribution and also z-potential were stable. The plasmid DNA content was also fairly stable changing no more than ~25 % during 6 months. Another standard way to store the liposome preparations is to freeze-dry the suspension in the presence of cryoprotectant (here sucrose at 5:1(w/w) to the lipid was used). Similarly to the storage in suspension the liposomes maintained their properties very well during 3 months storage in freeze-dried form at -20°C after rehydration.

As was mentioned above one of the major criteria is resistance of the liposomal preparation in the presence of serum. Obtained CCL-PEG suspension was incubated for up to 30 hours in the presence of 50% human serum. Next the liposomal fraction was separated from proteins on Sepharose 4B microcolumn and the diameter and z-potential measurements were performed. Incubation of these liposomes at 37°C in the presence of 50% human serum does not induce changes in the basic properties of CCL-PEG liposomes. To test the ability of the construct to preserve the encapsulated pDNA against serum DN-ases the electrophoretic analysis of the DNA was performed. The results suggest that the encapsulated DNA is preserved from DN-ase activities present outside liposomes, in solution.

The obtained carrier essentially did not show hemolytic activity when tested on freshly prepared human erythrocytes. The obtained values did not exceed 5% hemolysis after 30 min incubation with liposome suspension corresponding to ~25 mg lipid/ml. The observations based on Trypan Blue exclusion test indicate that proposed lipid carrier is essentially nontoxic to live cells of the tested cell lines (Jurkat T, HL-60 and K562) in PBS, culture medium or serum supplemented culture medium for 48 hours. Survival rate was similar for all 3 cell lines studied being close to 90% for the lower doses (0.15 mg lipid/ml) and 70-80 for the highest doses (0.75 mg lipid/ml) depending slightly on the medium in which cells were incubated (PBS, culture medium or serum supplemented culture medium). Animal (mouse) studies of fluorescently labelled CCL liposomes showed that more than 60% remained in the circulation for 6 hours and they could still be detected after 24 hours following injection. Control PE/PC (3/2) 100 nm in diameter liposomes were detected only for 3 hours.

Transfection efficiencies *in vitro* (cell culture) of the CCL-PEG liposomes were assessed by encapsulating pDNA encoding GFP or sometimes dsRED. The number of cells expressing fluorescent proteins were compared to the total number of cells. The observed efficacies were in the range of 60-80% showing rather limited dependence on time (24 or 48 hours) and on the dose, e.g. more than 65% for 0.25 mg pDNA/ml vs ~80% for 2.5 mg pDNA/ml.

Expression of *bcl-2* gene is a characteristic feature of most leukaemia cells, therefore the effect of transfection of the cells with CCL-PEG-encapsulated antisense ODN's was tested. The results of reverse transcriptase PCR analysis and Western Blot analysis of Jurkat T, K562 and HL60 cells treated with free and encapsulated in CCL-PEG for up to 72 hours indicate that mRNA signal decays within 48-72 hours after treatment with antisense ODN's containing CCL-PEG while marked decrease of the BCL-2 protein level can be observed after 24 of treatment. This may suggest that these ODN's affect strongly protein synthesis. To test if silencing of *bcl-2* gene has the effect on cell survival the experiment in which cultured cells were treated for 24 hours with increasing concentrations of encapsulated in CCL-PEG *bcl-2* directed antisense ODN's. The results indicate that after 24 hours of treatment with 2.5 mg/ml only ~40 of Jurtkat T and ~50% of HL60 cells survived. Data from these experiments indicate that the effect is dose dependent. When AML

patient's white blood cells were treated *in vitro*, in culture conditions with 1.5 mg/ml of CCL-PEG liposomes carrying antisense ODN's 40-50% of cells survived and the percentage of survival was proportional to the construct concentration.

In summary, results obtained from our experiments indicate that proposed lipid carrier is stable for at least 12 months during storage as a suspension at 4°C, and keep its properties for 3 months during storage in lyophilized form at -20°C. Moreover, our construct maintained relatively high level of encapsulated pDNA, protected it from external DN-ases and appeared essentially nontoxic against cells in the culture while not encapsulating specific ODN's. The CCL-PEG construct was stable in 50% human serum and showed high transfection efficiencies against human leukaemia cells. Moreover, when contained specific ODN's it was shown to effectively silence target (*bcl-2*) gene expression at the mRNA as well as protein level.

CONCLUSION

Gene therapy is a promising strategy for the treatment of many genetic diseases. The target of our study are cell blood cancer cells. We focused on acute leukemias cells represented by 3 cell lines Jurkat T, K562 and HL-60 that are known to be particularly difficult for transfection. The lipid carrier composition which was designed here was characterised with small particle size, good long-term (several months) stability in a suspension as well as in the form of freeze-dried powder. It was also stable for up to 30 hours incubated in the presence of 50% human serum. Moreover, it efficiently condensed and encapsulated pDNA and asODN. It also protected test preparations of pDNA from breakdown catalysed by external nucleases. The test CCL-PEG preparation did not induce substantial lysis of human erythrocytes and were not substantially toxic to the cultured cells of the studied here cell lines. They were also present in circulation for the extended time periods, characteristic for long-circulating liposomes when injected into mouse. By using this vector it was possible to transfect mentioned cell lines with high effectiveness reaching ~90%. This high level of transfection was also observed in the case of the same cells transfected with pDNA encoding siRNA's for other genes. Moreover, when *bcl-2* gene directed asODN were encapsulated the efficient silencing of this gene expression could be observed on either mRNA or protein level. This was accompanied by substantial toxicity of the proposed formulation to the cells in the culture and seems promissible for future studies on the combined asODN's-cytostatics chemotherapy.

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