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Review Article

In Vivo Characteristics of Cationic Liposomes as Delivery Vectors for Gene Therapy

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After a decade of clinical trials, gene therapy seems to have found its place between excessive ambitions and feasible aims, with encouraging results obtained in recent years. Intracellular delivery of genetic material is the key step in gene therapy. Optimization of delivery vectors is of major importance for turning gene therapy into a successful therapeutic method. Nonviral gene delivery relies mainly on the complexes formed from cationic liposomes (or cationic polymers) and DNA, i.e., lipoplexes (or polyplexes). Many lipoplex formulations have been studied, but *in vivo* activity is generally low compared to that of viral systems. This review gives a concise overview of studies on the application of cationic liposomes *in vivo* in animal models of diseases and in clinical studies. The transfection efficiency, the pharmacokinetic and pharmacodynamic properties of the lipid–DNA complexes, and potentially relevant applications for cationic liposomes are discussed. Furthermore, the toxicity of, and the induction of an inflammatory response in association with the administration of lipoplexes are described. Increasing understanding of lipoplex behavior and gene transfer capacities *in vivo* offers new possibilities to enhance their efficiency and paves the path to more extensive clinical applications in the future.

KEY WORDS: gene therapy; cationic liposomes; biodistribution; toxicity; inflammation.

INTRODUCTION

Using nucleic acids as drugs for gene therapy purposes has led to the development of sophisticated and efficient DNA carriers, also called vectors. The carrier should fulfill several minimal requirements, i.e., protection of the DNA from degradation before delivery, intracellular delivery to achieve gene expression, and safety. Vectors for gene therapy are usually classified as viral and nonviral, the latter being mainly represented by chemical methods of gene transfer. The choice of the delivery system is determined by the nature of the disease to be treated and the need for long-term vs. transient and low vs. high expression of the gene of interest. Although at present viral systems dominate in clinical trials for gene therapy, cationic liposomes have been studied in several clinical trials for treatment of cystic fibrosis, cancer, and, more recently, cardiovascular diseases (1).

The efficient transfection of eukaryotic cells using cationic liposomes was first described in 1987 by Felgner *et al.* (2). These cationic liposomes, composed of a cationic lipid {N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium-chloride, DOTMA} and a natural neutral phospholipid (dioleoyl phosphatidylethanolamine, DOPE) in a ratio 1:1, were shown to bind DNA efficiently, leading to cellular uptake of plasmid DNA and to high levels of transgene expression. Many synthetic amphiphiles have been synthesized since then that present the common features of vesicles forming in aqueous solutions, DNA binding, and more or less efficient gene transfer. Table I summarizes the cationic lipids mainly used for *in vivo* and clinical studies, classified according to structural properties. Cationic liposomes usually, though not always, include DOPE or cholesterol as a helper lipid.

Here, an overview of the present knowledge about the behavior of lipoplexes in animals and humans is given with a focus on therapeutic applications. This review does not go into the intracellular mechanism of transfection because this has been largely detailed elsewhere (3).

SYSTEMIC ADMINISTRATION OF LIPOPLEXES

Phamacokinetics and Biodistribution

Different methods (radiolabeled lipoplexes, PCR, Southern blot analysis) have been used to investigate the blood clearance and biodistribution of lipoplex components. These studies revealed that cationic lipid–DNA complexes are quickly cleared from the bloodstream when injected intravenously. Furthermore, the incorporated DNA seems to be rapidly subjected to degradation (4). Although organ distribution can be modulated by varying the lipid-to-DNA ratio or the size of the lipoplexes (5), lipoplexes usually accumulate, for the greatest part, in lung and liver and to a lesser extent in the spleen. However, the distribution between these two organs

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Table I. Cationic Lipids Described in the Text, Classified According to Structural Properties and Followed by References in Parentheses*

Hydrophilic part	Hydrophobic part	
	Double-chained lipids	Cholesterol-derived lipids
Monocationic	DDAB, DMRIE (4, 6, 22, 23, 26, 27), DODAC (30, 45), DOTAP (5, 9, 14, 32, 41, 45, 46, 55), DOTIM (7, 8, 15, 56), DOTMA (2, 10–12, 31, 45)	DC-chol (20, 25)
Polycationic	DOGS, DOSPA (41), DOSPER (24)	Lipid 67 (19, 33, 39, 42, 44)

* DDAB: dimethyldioctadecylammonium bromide, DMRIE: N-[1-(2,3-dimyristyloxy)propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium, DODAC: dioleyldimethylammonium chloride, DOGS: dioctadecyl amido glycyl spermine, DOSPA: 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N,-dimethyl-1-propanaminiumtrifluoroacetate, DOSPER: 1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propylamid, DOTAP: 1,2-dioleoyloxy-3-(trimethylammonio)-propane, DOTIM: 1-[2-(9(Z)-octadecenoyloxy)-ethyl]-2-(8(Z)-heptadecenyl)-3-(2-hydroxyethyl) imidazolinium chloride, DOTMA: N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride.

changes with time, implying redistribution. Lipoplexes are mostly found in the lung shortly after i.v. injection but eventually end up in the liver after 24 or 48 h (6,7). It has been suggested that the accumulation of lipoplexes in the lung could be explained by a first-pass effect. In the presence of serum proteins, lipoplexes could form aggregates, which would be captured in the first capillary bed encountered. However, fluorescently labeled 1-{2-[9(Z)-octadecenoyloxy]-ethyl}-2-[8(Z)-heptadecenyl]-3-(2-hydroxyethyl) imidazolinium chloride (DOTIM)- and 1,2-dioleoyloxy-3-(trimethylammonio)-propane (DOTAP)-based lipoplexes were shown to be taken up by endothelial cells in the lung (8,9), whereas in the liver Kupffer cells were responsible for lipoplex uptake (8).

Pattern and Characteristics of Transgene Expression

In accordance with the distribution of the lipoplexes, the highest level of expression of the transgene is obtained in the lung with most formulations. In contrast, uptake by the liver generally leads to degradation and thus to low expression levels. Depending on the cationic lipid formulation, significant expression could also be detected in the heart, kidneys, spleen, and liver after i.v. injection of lipoplexes (10,11). Expression was also reported in lymph nodes (12) and in thymus and colon (5) when DOTIM and DOTAP were used as the cationic lipid, respectively.

Identification of the cells transfected in the lung led to contradictory observations. Transgene expression in airway and alveolar parenchymal cells was described by Zhu *et al.* (12), whereas others showed that mostly endothelial cells and some interstitial macrophages expressed the transgene (11,13). Using fluorescently labeled lipids, McLean and colleagues (8) demonstrated that lipoplexes could extravasate only in the spleen. The discontinuous endothelium in this organ could provide an explanation for this finding. Although main features are shared by the lipoplexes used in the studies mentioned above, subtle differences in outcome may be partly explained by both the nature of the cationic and helper lipids and the mode of lipoplex preparation.

Mechanism of Transfection After i.v. Administration

The events leading to *in vivo* lipofection remain largely unclear, and only a few studies have addressed this issue. Naked DNA is rapidly degraded when injected intravenously, clearly demonstrating the protective role of cationic liposomes. However, it is likely that cationic liposomes also play an active role in the intracellular delivery (14). Possibly, proteoglycans exposed at the cell surface mediate lipoplex–cell binding in the pulmonary vasculature (15), which is followed by internalization of the particles within 1 h (16). Whether a specific receptor is also involved at this stage is not yet known. Although differences may exist depending on the transgene used, protein expression in the lung can often be detected within the first hours following injection (10,11) and rapidly decreases after 24 h or a few days. Proposed explanations for transient transgene expression are degradation of the plasmid DNA, inhibition of expression by other factors such as cytokines (see below), or toxicity, as suggested by the detection of apoptosis in the lung as a result of lipoplex administration (see below). Furthermore, transgene expression after a second administration of lipoplexes is subject to a refractory period of about 2 weeks, meaning that this span of time is needed to get levels of expression similar to the one following the first injection (11,17,18). This temporal inhibition has been related to the production of antiinflammatory cytokines following the first injection. These cytokines are able to shut down the viral promoters usually placed before the transgene (see below). The lack of sustained transgene expression and the interval required between two administrations may be important limitations to therapy and therefore need to be taken into account in the design of treatment schedules.

Further investigations to unravel the course of spatiotemporal transfection *in vivo*, in relation to the cell type expressing the transgene as well as the nature of the transgene and the promotors used, are essential for improvement of transfection efficiency and specificity, an obvious requirement for clinical applications.

LOCAL ADMINISTRATION OF LIPOPLEXES

Local administration of lipoplexes, when feasible, could circumvent some of the problems associated with systemic administration. This route allows organ-restricted expression, avoiding the need to retarget the lipoplexes, and should reduce side effects such as toxicity toward nontarget tissues. Several organs have been exposed to local delivery in order to examine and characterize gene expression and/or to test therapeutic approaches. These studies point out that gene therapy strategies for local delivery also have to surmount various hurdles, as discussed below.

Intratumoral Administration

A recent study compared various delivery routes for transfection of an intrahepatic tumor with plasmid DNA encoding chloramphenicol acetyltransferase (CAT) complexed by GL-67-based cationic liposomes. Intratumoral injection re-

sulted in 2000- and 250-fold increases of protein expression over those evoked by intravenous or intraportal administration, respectively, clearly demonstrating the advantage of local delivery (19). In a subcutaneous melanoma model, expression of the same reporter gene (CAT) following intratumoral administration of DC-chol-based lipoplex was restricted to the injection site because of the poor diffusion capacity of these particles (20). Furthermore, in subcutaneously grown tumors, naked DNA sometimes led to better transfection than lipoplexes (21), but the opposite was also found (22). This apparent contradiction may be explained by the different tumors and cationic lipids used, but parameters such as lipid:DNA ratio may also be of relevance for the efficacy of the lipoplexes. Therefore, transfection protocols using DNA–lipid complexes should be optimized for each tumor model and lipoplex formulation. This implies characterization of the lipoplexes with regard to size and possibly surface potential. Careful studies of transfection comparing different tumor models using one liposomal formulation or different cationic lipids in one model will also provide useful information in this respect.

Factors governing local gene expression after intratumoral delivery have been investigated by Clark *et al.* (22), who showed that lipid:DNA ratio and DNA dose were important factors determining expression level. In contrast, injection volume or technique, i.e., a single injection in the center of the tumor or multiple injections throughout the tumor, were of little influence. Other studies also indicated that the net charge of the lipoplex is an important parameter, with a negative net charge favorable for high expression (23).

In various tumor models, therapeutic benefit was tested after intratumoral administration of lipoplexes containing genes encoding immunomodulators (e.g., IL-2, IL-4, IL-12, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IFN-β), suicide genes (e.g., herpes simplex virus thymidine kinase), or a bacterial toxin (24). These studies generally showed high percentages of antitumor responses, from tumor growth delay to complete remission, thereby confirming the therapeutic potential of these strategies. In some cases an intriguing nonspecific antitumor response was reported (25,26). This response consisted of transient tumor regression on treatment with an empty plasmid (i.e., without the therapeutic gene). This antitumor effect is possibly based on the cytotoxicity of the injected formulation or on nonspecific immunostimulation by the DNA present in the lipoplexes (see below).

In the clinic, promising results were obtained by lipidic intratumoral delivery of the HLA-B7/β_2-microglobulin genes. The expression of this allogeneic MHC molecule by tumor cells should enhance their immunogenicity and thereby trigger tumor elimination by the immune system. The efficacy of this strategy appeared to be dependent on the type of cancer. A similar tumor-type dependence was observed for IL-2 antitumor gene therapy, with a higher percentage of responses in patients with renal cell carcinoma compared to melanoma or sarcoma (27).

Administration to the Lung

Although the lung is the first organ showing transgene expression following i.v. injection, this organ is also easily accessible for local delivery. Therefore, the possibility of administering lipoplexes in an aerosol formulation was investigated (28). Whereas this approach circumvents the endothelial barrier, it also presents several obstacles for gene delivery using lipoplexes. The lipoplexes first encounter an epithelium of polarized cells that are quite refractory to lipofection. Furthermore, the observation that surfactants inhibit *in vitro* transfection by lipoplexes suggests that lung surfactants may represent a barrier to transfection *in vivo* (29). Nevertheless, transfection was reported on pulmonary administration of lipoplexes with DNA encoding luciferase, β-galactosidase, or CAT. Higher levels of transgene expression were obtained using lipoplexes compared to naked DNA (30). In an animal model for cystic fibrosis (CF), correction of the CF-associated defects was demonstrated after intratracheal delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (31).

In patients, no clinical benefit of CFTR-gene therapy has been demonstrated yet, but proof of the principle was obtained for expression of the CFTR gene as a functional protein in the nasal airway of CF patients after lipoplex treatment. The transgene expression was accompanied by partial correction of the ion transport defect associated with the disease (32). These trials also demonstrated the safety of the lipoplex treatment because no alteration of lung function, no local or systemic inflammation, and no changes in clinical chemistry and blood profiles were observed. Similar findings regarding safety and efficacy were made following delivery to the lung by nebulization of the lipoplexes (33).

Therapeutic outcome might be further improved by optimization of the cationic lipid systems with regard to lipoplex internalization for pulmonary transfection. To this end, a targeting device could be attached to the lipoplexes, as suggested by Scott *et al.* (34). They showed enhanced transfection of polarized cells *in vitro* by integrin targeting via an RGD peptide compared to the nontargeted system. Lung transfection is a relevant issue for gene therapy because not only cystic fibrosis but also other diseases such as α_1-antitrypsin deficiency could be of interest for this application (35).

Other Indications for Local Lipoplex Administration

Genetic vaccination is a promising strategy for treatment of infectious and malignant diseases. Intramuscular and intradermal administration of naked DNA can raise a specific immune response against the protein encoded by the transgene. Although efficient immunization has been obtained by needle injection or particle bombardment of naked DNA, recent reports suggest that cationic liposomes could also prove beneficial for this purpose. After intramuscular injection, lipoplexes enhanced the humoral (36) and cellular (37) responses obtained with naked DNA encoding viral proteins (influenza nucleoprotein and hepatitis B surface antigen, respectively). Furthermore, lipoplexes were particularly efficient in inducing mucosal immunity by nasal delivery compared to naked DNA (38). The gain of specific immunogenicity when lipoplexes are used instead of naked DNA may (partly) result from higher expression levels of the antigen. Alternatively, enhanced delivery of the transgene to cells active in immunostimulation such as dendritic cells may be the basis for this observation.

Despite obvious advantages, local application of lipoplexes is not always satisfactory regarding transfection efficiency, and it is occasionally associated with toxicity and inflammation as described in the following section.

LIPOPLEX-INDUCED TOXICITY

Intravenous injection of cationic lipid–DNA complexes is often accompanied by a dose-dependent toxicity. In the days following injection, piloerection and lethargy were observed in the treated mice. Clinical analyses have shown a drop in the number of circulating lymphocytes (lymphopenia) and an increase in the serum levels of liver enzymes, which indicates liver damage (39). Hepatic necrosis was observed by microscopic analysis (11,40). Usually these symptoms regressed back to normal within a few days after treatment, although several formulations were reported to be lethal above a certain dose at high lipid:DNA ratio (11). Severe liver damage could be the cause of the mortality. It should be noted that, when injected alone, the components of the lipoplexes did not display any toxicity, even at much higher doses, suggesting that the damages relied on either the structure of the lipid–DNA complexes or lipoplex-associated features. Lung toxicity was also noticed after i.v. injection as reflected by apoptosis of endothelial cells (18), and local lung delivery induced the production of reactive oxygen intermediates (41).

Recently inflammation was reported in cystic fibrosis patients after lipoplex aerosolization (42), an aspect that is further detailed in the next section. In general, the toxicity associated with lipoplexes presents a limit to the large-scale use of these complexes, and the basis of this toxicity remains to be elucidated. Because the toxicity associated with lipoplexes may be species dependent, preclinical testing of lipidic formulations on both nonhuman and human organ slices could give valuable information related to this important issue (43).

LIPOPLEXES ACTIVATE INNATE IMMUNE RESPONSES

Characteristics of Lipoplex-Induced Immune Responses

Cationic lipid–DNA complexes are potent activators of the innate immune system. Several studies have investigated this activity and its consequences at different levels, notably transgene expression and antitumor effects. Immune activation could be seen after local (44,45) and i.v. administration (46) and was characterized by the systemic release of proinflammatory cytokines such as IL-6, IL-12, TNF-α, and IFN-γ. Infiltration of T cells and NK cells was evidenced in the lung (47). In marked contrast to the production of cytokines induced by lipoplexes, the cell influx was also seen with cationic liposomes without DNA (48). To a certain extent, these responses depended on the synthetic amphiphile used but did not seem to be restricted to lipoplexes, as they were also observed with polyplexes based on (poly)ethyleneimine (49). No relationship between lipid structure and intensity of the response has been established yet. The trigger of this nonspecific immune cascade seems to be the bacterial origin of the plasmid DNA, incorporated in the lipoplexes. Bacterial DNA differs from eukaryotic DNA by the higher frequency of CpG motifs and hypomethylation of these motifs. As a result, it is a well-known, potent stimulator of B cells, especially macrophages, and thereby induces, among others, the production of proinflammatory cytokines (50). In support of this hypothesis, methylation of the plasmid DNA by enzymatic reaction before systemic delivery led to a reduced release of cytokines (18,46,47). The data so far suggest that the events in this process take place as depicted in Fig. 1.

Consequences of Immune Activation by Lipoplexes

On local and systemic administration, lipoplexes can activate the innate immune system. In the presence of a preexisting inflammation, systemic administration of lipoplexes increased the mortality and the severity of symptoms seen in these animals (51). The effect of cytokine release on the transfection efficiency of lipoplexes consisting of protamine–DOTAP:cholesterol liposomes and plasmid DNA encoding luciferase was studied by Li *et al.* These authors showed that the production of TNF-α and IFN-γ was responsible for low levels of transgene expression in the lung and for inefficient transfection when a second injection was performed shortly after the first one (18). It was hypothesized that the production of TNF-α led to apoptosis of lung endothelial cells, resulting in low transfection efficiency and inactivity on a second injection. A direct effect of the cytokine release may also be considered, as TNF-α and IFN-γ were shown to exert a direct inhibitory effect on transfection *in vitro*, at the level of cellular uptake of the particles (52), and of mRNA production (53). Indeed, the viral promoters used in most DNA vectors (CMV, SV40, RSV) are known to be susceptible to inhibition of gene transcription by TNF-α and IFN-γ. An indirect effect of the cytokines is likely involved as well, considering the lack of correlation between time course of cytokine production and duration of the inhibition (46).

Methylation of the DNA (provided that this does not interfere with expression of the transgene) and the use of nonviral promoters are in this respect optional when immune activation is undesirable. Sequential i.v. injection of cationic liposomes and naked DNA reduced toxic and immunologic

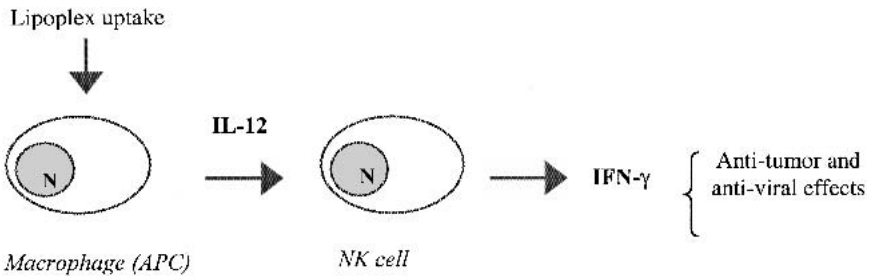

Fig. 1. Mechanism of immune activation on lipoplex delivery. Following uptake of lipoplex, antigen-presenting cells (APC) are activated by the bacterial DNA present in the lipoplexes and respond by the production and release of, among others, IL-12. IL-12 is a potent activator of NK cells that, on stimulation, release IFN-γ. N = nucleus.

responses without affecting expression levels (54) and thus represents another alternative to avoid negative effects.

Potential Advantages of Immune Activation by Lipoplexes

Proinflammatory responses on lipoplex administration could be advantageous in case of vaccination or antitumor immunotherapies. After i.v. injection of cationic lipid–DNA complexes, regression of lung metastases was observed even when the plasmid DNA contained no therapeutic gene (55). This "empty vector effect" relied on the ability of lipoplex to elicit the release of cytokines and activation of NK cells (46,47) and enhanced the specific antitumor response when the IL-2 or IL-12 gene was included in the lipoplexes (47). A gene-dependent antitumor response mediated by cationic lipid–DNA complexes was also reported (56), suggesting that this effect also depends on immunogenicity of the tumor itself. As mentioned above, intratumoral delivery of lipoplex with noncoding DNA also occasionally led to therapeutic benefit. A possible explanation, which deserves further investigation, could be the local activation of immune responses, as suggested by the cytokine production and T-cell infiltration noticed in two studies following injection of nonrelevant DNA-containing lipoplexes (21,57). Immune activation could also be favorable for vaccination against infectious agents. In this particular case, the DNA could be considered as an adjuvant and enhance the specific response to the antigen encoded in the DNA. Cationic liposomes may contribute to this effect by protecting the DNA molecule, increasing its uptake by antigen-presenting cells, or favoring its intracellular processing for presentation.

Immune Response–Related Toxicity

The possible link between the strong cytokine response and the clinical toxicity symptoms caused by lipoplex administration was investigated by Toussignant *et al.* They showed that leukopenia and liver damage did not correlate with the production of TNF-α (39). In contrast, methylation of the DNA could reduce liver toxicity (40), which suggests involvement of an immune factor. Furthermore, reducing the inflammatory reaction on lipoplex administration decreased the intensity of toxic symptoms (54). Whether toxicity and inflammatory responses associated with the use of lipoplexes are related should be elucidated in order to decrease or modulate their effect in an independent way. This could facilitate the development of safer carrier systems for clinical applications that would be less toxic and nonimmunogenic or more immunogenic when this could positively contribute to the treatment.

CONCLUSIONS

The behavior of lipoplexes *in vivo* has been the object of many studies in recent years. Increasing insight has been obtained with respect to tissue distribution, parameters governing transfection efficiency, toxicity, and elicited immunogenicity. However, differences in transfection efficiency and toxicity are observed depending on the route of administration. The cellular and molecular mechanisms behind *in vivo* transfection should be elucidated to understand these differences. This knowledge will set the basis for further improvement to allow optimization of the formulations and of treatment regimens.

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