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Review

Cationic Lipid-Based Gene Delivery Systems: Pharmaceutical Perspectives

Ram I. Mahato,[1,2] Alain Rolland,[1] and Eric Tomlinson[1]

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Gene delivery systems are designed to control the location of administered therapeutic genes within a patient's body. Successful *in vivo* gene transfer may require (i) the condensation of plasmid and its protection from nuclease degradation, (ii) cellular interaction and internalization of condensed plasmid, (iii) escape of plasmid from endosomes (if endocytosis is involved), and (iv) plasmid entry into cell nuclei. Expression plasmids encoding a therapeutic protein can be, for instance, complexed with cationic liposomes or micelles in order to achieve effective *in vivo* gene transfer. A thorough knowledge of pharmaceutics and drug delivery, bio-engineering, as well as cell and molecular biology is required to design optimal systems for gene therapy. This mini-review provides a critical discussion on cationic lipid-based gene delivery systems and their possible uses as pharmaceuticals.

KEY WORDS: non-viral gene delivery; plasmid; cationic liposomes; formulation; transfection.

INTRODUCTION

The aim of somatic gene therapy is to provide specific cells of a patient with the genetic information necessary to produce therapeutic proteins for correction or modulation of diseases. Gene therapy methods are designed for both improving the delivery of existing therapeutic proteins that have inappropriate pharmacokinetic profiles and treating diseases that currently are considered untreatable with protein drugs. Gene therapy can also be used for improving intracellular trafficking of therapeutic proteins to different cell organelles that are not effectively targeted by extracellular administration of proteins or to achieve therapeutic effects of low molecular weight drugs, such as prostaglandins or nitric oxide, whose synthesis may be induced *in vivo* by overexpression of rate-limiting synthetic enzymes (1).

In general, a non-viral gene medicine is composed of three elements: a gene encoding a therapeutic protein, a synthetic delivery system that controls the location of the gene within the body and a plasmid-based gene expression system that controls the function of a gene within a target cell (2). The development of cationic lipid-based gene medicines requires careful consideration of the potency of plasmid, target selectivity and toxicity of lipids, bioavailability of plasmid/lipid complexes and the possibility to manufacture them on a large scale. Plasmids represent an attractive new class of pharmaceuticals. However, the use of plasmid-based gene expression systems as "pre-drug" molecules is limited by their colloidal and surface properties, as well as by their susceptibility to nucleases. Plasmids are large, hydrophilic macromolecules (~3,000 kDa) with a net negative surface charge. Their size and charge density depend on the numbers of base pairs and DNA conformation. Supercoiled DNA has been shown to have a higher negative charge density than linear or nicked-circular species, and consequently different plasmid forms have different ζ potentials ranging from -30 to -70 mV (2). These characteristics prevent plasmids from crossing biological membranes efficiently. A fundamental challenge for the effective targeted delivery of plasmid-based gene expression systems is to control the surface and colloidal properties of plasmids in a biological environment, since these properties influence their biological distribution, cellular uptake, intracellular trafficking and nuclear translocation (3,4).

Since the introduction in 1987 of the transfection reagent Lipofectin™, a 1:1 w/w mixture of the cationic lipid DOTMA (N[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) and the colipid DOPE (dioleylphosphatidylethanolamine) (5), many cationic lipid formulations have been tested for *in vitro* and *in vivo* transfection of nucleic acids. Although *in vivo* transfection efficiency of the current cationic lipid-based gene delivery systems is generally lower compared to viral vectors, this approach has proven useful in many *in vivo* applications in animal models (6–8) and more recently in human clinical trials (9,10). The flexibility in the design of cationic lipid structure and liposome composition, coupled with the diversity of methods for their preparation and *in vivo* efficiency, have promoted the notion that cationic lipids can be efficiently used in human gene transfer. In this mini-review, we will present different cationic lipids from the viewpoint of gene delivery and formulation, with an emphasis on pulmonary and hepatic gene delivery.

[1] GENE MEDICINE, INC., 8301 New Trails Drive, The Woodlands, Texas 77381-4248.

[2] To whom correspondence should be addressed. (e-mail: mahatr@genemedicine.com)

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PLASMID-BASED GENE EXPRESSION SYSTEMS

Plasmid-based gene expression systems contain a therapeutic gene and other DNA sequences to control the *in vivo* production of a protein. They may contain genetic sequences that control the level of transcription and consequently produced protein in a cell-, or disease-specific manner. Expression systems also may contain elements that control the stability of mRNA and the timing of protein production. Such sequences may include cell-specific promoters and enhancers to restrict gene expression to specific sites within the body (3). The most commonly used viral promoter/enhancer region is the human cytomegalovirus (CMV) immediate-early gene promoter/enhancer. This regulatory region provides the highest levels of gene expression in a variety of cell types and tissues of most species. The expression plasmid may also contain transcript stabilizers that prolong the stability of transcribed mRNA and consequently the level and duration of protein production. Specific introns and polyadenylation sequences can be included in the gene expression systems to increase the production of therapeutic proteins in specific tissues and ensure fidelity of gene expression. Introns are usually placed immediately downstream of the promoter/enhancer region. Placement of an appropriate 3′ untranslated region (UTR) immediately downstream of the cDNA coding region is important, since it provides for mRNA stability, efficient transport of mRNA to the cytoplasm and can increase the efficiency of mRNA translation. Genetic sequences located at the 5′ and 3′ end of the coding region can be selected to direct translation of mRNA, post-translational processing and secretion of the protein. Gene switches can optionally be included in a gene expression system to enable the function of an administered gene to be controlled, i.e., turned on or off, using low molecular weight drugs (11).

CATIONIC LIPID STRUCTURES AND COMPLEX FORMATION

Cationic lipids are amphiphilic molecules that interact with the negatively charged phosphate backbone of DNA, neutralizing the charge and promoting the condensation of DNA into a more compact structure. Cationic lipids are typically employed in combination with a neutral and zwitterionic colipid such as DOPE (5,12,13) or cholesterol (Chol) (14). However, inclusion of a colipid is not always essential. For instance, the cationic lipid 1,2-bis (oleoyloxy)-3-(trimethylammonio)propane (DOTAP) is active in the absence of a colipid in a variety of cells *in vitro* (15). The cationic amphiphiles differ markedly and may contain single or multiple charges (primary, secondary, tertiary and/or quaternary amines), cationic cholesterol derivative, cationic detergent or lipopolylysine. Some of the cationic lipids currently being investigated for gene transfer include, for instance, DOTMA, dimethyl dioctadecylammonium bromide (DDAB), dioctadecylamidoglycylspermine (DOGS), DOTAP, N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide-(DMRIE), 2,3-dioleyloxy-N-[2(sperminecarboxyamido)ethyl]-N,N-dimethyl-1-propanaminium trifluroacetate (DOSPA), 3β[N-(N′,N′-dimethylaminoethane)-carbamoyl)] cholesterol (DC-Chol), 1-[2-(9(Z)-octadecenoyloxy ethyl]-2(8-(Z)heptadecenyl-3-hydroxyethylimidazolium chloride (DOTIM), (+/−)N(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide (GAP-DLRIE) and 3-β-(N^4-spermine carbonoyl)cholesterol (lipid #67) (5,7,15–23).

The three basic parts of cationic lipids include (i) a *hydrophobic lipid anchor group* which helps in forming liposomes (or potentially micellar stuctures) and can interact with cell membranes, (ii) a *linker group*, and (iii) a *positively charged headgroup* which interacts with plasmid, leading to its condensation (Fig. 1). The linker group is an important component which determines the chemical stability and biodegradability of the lipid. The acyl chains of DOTMA and DMRIE are, for instance, connected to the cationic headgroup by more stable ether linkages, compared to the labile ester linkages of DOTAP. The physicochemical properties of plasmid/lipid complexes are strongly influenced by the relative proportions of each component, and the structure of the headgroup. Many effective cationic lipids contain protonatable polyamines linked to dialkyl or cholesterol anchors. Cationic lipids with multivalent headgroups have been shown, in general, to be more effective for gene transfer when compared to their monovalent counterparts (7,19).

Although cationic lipid-based gene delivery systems are being intensively investigated and novel cationic lipid molecules are synthesized routinely, no definite structure-activity relationship has clearly emerged so far. Lee *et al.* (23) have recently attempted to establish some structure-activity relationships by systematically analyzing a large number of different cationic lipid structures both *in vitro* and *in vivo*. Cationic lipids containing 3-β-(N^4-spermine carbamoyl)cholesterol (lipid #67) and 3-β-(N^4-spermidine carbamoyl)cholesterol (lipid #53) in a "T-shape" configuration rather than a linear configuration were found to be more active than structures harboring only a single protonatable amine (e.g., DC-Chol). However, there was a poor correlation between *in vitro* and *in vivo* results with various lipid systems used in this study. Although the cationic lipids DOGS and DOSPA also contain spermine headgroups, they were less active than the cationic lipid #67, possibly due to the following differences in their structures: (i) the headgroup of lipid #67 is attached to the linker via a nitrogen atom, whereas those of DOGS and DOSPA are attached through a carbon atom; (ii) both DOGS and DOSPA harbor dialkyl chain as their lipid anchor groups, whereas #67 harbors cholesterol anchors; and (iii) lipid #67 is in a free base form, whereas DOGS and DOSPA are in the salt forms.

PHYSICOCHEMICAL PROPERTIES

Plasmid/lipid complexes often aggregate over time in the presence of salt or serum proteins. Increasing ionic strength induces aggregation, in particular in the presence of polyanionic salts, such as phosphate and succinate. Plasmid/lipid complexes also tend to precipitate at high plasmid concentration. Electri-

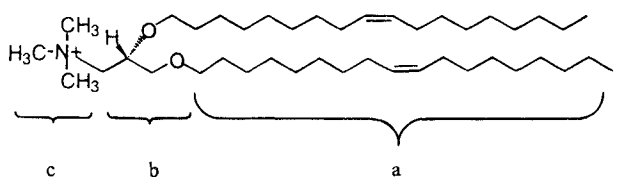

Fig. 1. Basic components of a cationic lipid (DOTMA). a, hydrophobic lipid group; b, linker group; c, cationic headgroup.

cally neutral plasmid/lipid complexes are difficult to prepare since they are prone to aggregation. Aggregation of plasmid/lipid complexes is a function of cationic lipid species, DNA/cationic lipid ratio, DNA and lipid concentrations, shearing force, temperature, solution viscosity and time (24,25). It can be prevented by controlling the ratio of DNA to liposomes, minimizing the overall concentration of plasmid/lipid complexes in solution, and avoiding the use of chelating agents such EDTA and/or significant amounts of salts.

Due to their colloidal instability, plasmid/lipid complexes have often been formed immediately prior to administration to subjects or addition to cultured cells. This has limited the ability to standardize, characterize, and control the quality of these formulations. Therefore, methods for lyophilizing the lipid-based systems in the presence of specific cryoprotectants have been developed to increase the stability of plasmid/lipid complexes. The cryoprotectants prevent aggregation and fusion of plasmid/lipid complexes during lyophilization cycles. Following lyophilization and rehydration to isotonicity, physicochemical characteristics of plasmid/lipid complexes (particle size, ζ potential and complexation efficiency) and transfection efficiency of the lyophilized complexes remained unchanged (26).

The particle size and ζ potential of plasmid/lipid complexes depend on i) the cationic lipid species, ii) amount and type of the colipid, iii) the particle size and composition of liposomes used in the complexation, iv) molecular weight, form and type of plasmid, v) the stoichiometry of cationic lipid and DNA, v) the concentration of cationic lipid and DNA in the final formulation, and vi) mixing procedures (1–8) (Table 1).

Particle morphology of plasmid/lipid complexes has been studied by Gershon *et al.* (27) under transmission electron microscopy (TEM). They proposed that at low ratios of cationic lipid to DNA, positively charged vesicles are adsorbed through electrostatic interactions to the polynucleotide to form aggregates that gradually surround larger segments of the DNA. As the amount of lipids is increased, the aggregated liposomes along the DNA reached a critical concentration at which lipid fusion and cooperative DNA collapse processes are initiated. Following an additional increase of the lipid concentration, the collapsed DNA structures become coated by the lipid bilayers, leading to efficient DNA condensation. Thus, a very slight increase of the liposome concentration above the critical value required for DNA collapse is sufficient to complete the condensation process. This results in coating of DNA with a lipid bilayer along the entire length of the plasmid.

Under freeze-fracture electron microscopy, the complexes made of DNA plasmid, a monovalent cationic amphiphile, such as DC-Chol, DOTMA, DOTAP and DMRIE, and a neutral lipid DOPE showed 'spaghetti-meatball' structure formation. In contrast, neither fusion nor spaghetti formation was observed during interaction of DNA plasmid with liposomes made of polyvalent cationic amphiphile DOSPA (28). However, further investigation is required to determine whether the observed morphological variations are due to the structural differences in their cationic headgroups and linker bonds or due to the preparation process of DOSPA-based formulations. Positively charged plasmid/DOTMA:DOPE complexes have been reported to have a monodispersed size distribution and consistent 'fingerprint' structure, with no apparent free plasmid, whereas their negatively charged counterparts were polydispersed and contained a large amount of free DNA (2). At 1:3 (−/+) charge ratio, plasmid/DOTMA:DOPE complexes formed discrete spherical multilamellar particles, whereas using a different co-lipid, 'ball and string' structures which are similar

Table 1. Influential Factors on Cationic Lipid-Based Gene Delivery Systems

Characteristics	Affecting factors
1. *Physicochemical characteristics*	
size, shape, charge and stability:	ionic strength, lipid composition, lipid/DNA ratio and concentration, mixing procedures
2. *Transfection*	
DNA plasmid:	physical form, promoter/enhancer, cDNA and introns
cationic lipid:	lipid anchor, linker and cationic headgroup
target organ:	mode of delivery and degree of vascularization
adhesion to cell surfaces:	adsorption or recognition by receptors
cellular internalization:	endocytosis, fusion or entry through cell membrane pores
DNA release:	endosomal, lysosomal or cytoplasmic
nuclear trafficking:	passive diffusion, active transport or fusion
3. *In vivo characteristics*	
stability:	plasma or other biological milieus
pharmacokinetics/biodistribution:	target organs vs. non-target organs, plasma half-life, excretion rate
efficacy:	level and duration of gene expression
toxicity:	cellular and systemic
4. *Manufacturing issues*	
reproducibility:	aggregation vs. stable colloidal suspension
sterility	
impurity:	endotoxin, chemical degradation
shelf-life stability:	suspension vs. lyophilized products
scalability	

to that observed by Sternberg *et al.* (28) were observed (Duguid et al., unpublished data).

INTERACTION WITH BIOMOLECULES

In vitro transfection with cationic lipids is generally best obtained when plasmid/lipid complexes bear a strong positive charge. However, positively charged complexes may interact with serum proteins, lipoproteins, heparin and glycosaminoglycans in the extracellular matrix, leading to the aggregation or release of DNA from the complexes even before reaching the target cells. The poor correlation between *in vitro* and *in vivo* transfection activities of plasmid/lipid complexes may be in part due to the premature release or aggregation of DNA *in vivo* due to a different biological environment (29). Cationic lipids activate the complement system, and thus intravenously administered plasmid/lipid complexes are opsonized by C3b/C4b components, leading to their rapid clearance by macrophages in the reticuloendothelial systems. Activation of the complement system will decrease the plasma half-life of plasmid/lipid complexes and thus these complexes might be cleared from the circulation before they have any chance to interact with their targets [30]. However, some authors have observed weaker activation of the complement systems by plasmid/lipid complexes compared to plasmid/poly(L-lysine) complexes. It has been proposed that opsonization may also retarget plasmid/lipid complexes and influence gene expression patterns in various organs governed by the presence or absence of complement receptors in various tissues (31). The preparation of negatively charged plasmid/lipid complexes or surface modification of these complexes with a steric stabilizer such as polyethyleneglycol (PEG) are likely to minimize and possibly avoid the activation of the complement systems.

TARGETED GENE DELIVERY

Cationic lipid-based systems lack target specificity which results in low transfection efficiency in certain tissues due to the interference from cationic lipid-binding macromolecules either circulating or in the extracellular matrix. The electrostatic interaction between the positively charged plasmid/lipid complex and the cell membrane is usually so overwhelming that inclusion of any targeting ligand is usually not able to provide cell-specificity. To circumvent this problem, neutral plasmid/lipospermine complexes containing a trigalactolipid have been prepared and shown to efficiently transfect hepatoma HepG2 cells bearing asialoglycoprotein receptor. Addition of 25% (mol/mol) of the triantennary galactolipid increased the transfection efficiency by a thousand fold as compared to the lipid-based system with no targeting ligand (32). Recently, the combination of transferrin and cationic liposome Lipofectin™ showed efficient transfection of β-galactosidase into HeLa cells, whereas Lipofectin™ alone had low transfection efficiency (33).

IN VIVO HEPATIC AND PULMONARY GENE EXPRESSION

The colloidal and surface properties of plasmids determine their biodistribution and cellular uptake (3,4). They do not efficiently cross intact biological barriers, such as continuous capillary endothelium, keratinized epithelium, mucosal epithelium or the blood-brain barrier. In addition, the penetration of a plasmid into a cell without transient permeabilization of the plasma membrane and/or disruption of the endosomal membrane following endocytosis, represents a very inefficient process. The diffusion of plasmid through extracellular matrices, such as the connective tissues in skeletal muscle, is also very limited (3).

Bioavailability of plasmid/lipid complexes to target tissues may be limited by their large particle size and possible aggregation and/or dissociation in physiological fluids. For example, particulates are rapidly removed from the bloodstream after systemic administration by cells of the mononuclear phagocyte system, mainly Kupffer cells in the liver. This process has been shown to result from the interaction of the injected particulates with opsonins. Therefore, preferential gene delivery to Kupffer cells can be achieved by controlling opsonization of the formulated plasmid/lipid particles. However, effective hepatocyte gene delivery would require particulate systems with characteristics not currently observed, including colloidal stability in blood and particle size below 100 nm for extravasating through the sinusoidal hepatic endothelium and then passage through the space of Disse (33). Although a large percentage of injected plasmid/lipid complexes are taken up by the liver, gene expression in this organ often remains low (34,35), as plasmid/lipid complexes are largely taken up by Kupffer cells via phagocytosis, which presumably results in degradation of the DNA and inefficient gene expression (36). However, gene expression in the mouse liver has shown to be increased significantly by intravenously injected plasmid/lipid complexes prepared using DOTIM:Cholesterol (1:1 molar ratio) multilamellar vesicles (MLV), possibly by increasing the tissue deposition and subsequent retention of the expression plasmid (37).

The lung is one of the most attractive target organs for cationic lipid-based gene delivery, as plasmid/lipid complexes can be delivered to pulmonary endothelium by intravenous administration (38) as well as to epithelium and alveolar macrophages by intratracheal instillation or aerosol administration (39). The particle size, ζ potential and hydrophobicity of the complexes may be controlled to facilitate the ionic and/or hydrophobic interaction of the particles with the lung mucosa as well as vascular endothelium to achieve effective *in vivo* gene transfer (1).

Both intratracheal and aerosol delivery methods bypass the endothelial barrier and allow direct access of genes to the respiratory epithelial cells. Numerous genes including chloramphenicol acetyl transferase, human α1-antitrypsin, and cystic fibrosis transmembrane conductance regulator genes have been expressed in the lungs after pulmonary administration of plasmid/lipid complexes (39–41). Compared to intravenous administration, intratracheal instillation of plasmid/lipid complexes shows higher and more prolonged gene expression in murine lung. Immunohistochemical and histochemical analysis of lung tissues after intratracheal administration of plasmid/lipid complexes have shown that gene products are present in the airway epithelial cells (22,42).

Since intratracheal instillation is not a likely route for clinical administration of plasmid/lipid complexes to the lung, aerosol delivery has been used to demonstrate successful cationic lipid-mediated gene delivery. Prolonged gene expression (up to 21 days) in both bronchiolar and alveolar epithelial cells has been achieved using plasmid/DOTMA:DOPE complexes delivered by aerosolization (43). Optimal *in vivo* aerosol deposi-

tion in the respiratory tract requires aerosols of specific mass median aerodynamic diameter (MMAD) of 1 ~ 5 μm. Aerosolization of free plasmids causes excessive degradation, whereas plasmid/lipid complexes withstands the shearing force during nebulization.

MECHANISM OF GENE TRANSFER

Cationic lipid-based gene delivery systems rely on cellular mechanisms to deliver DNA into the cytoplasm and to the nucleus for gene expression. Both DNA condensation and dissociation from plasmid/lipid complexes at the correct site are crucial to obtain high level of gene expression. The major cellular barriers for efficient gene transfer are schematically illustrated in Fig. 2. After being delivered to the cell surface, plasmid/lipid complexes must gain access to the intracellular compartment and find their way to the nucleus. The cellular uptake is a relatively inefficient process and once inside the cell, the plasmid/lipid complex has no inherent mechanism to deliver the DNA to the nucleus (44). In the absence of cell division and nuclear membrane disruption, entry of plasmids into the nucleus through the nuclear pore complex remained an unexplained phenomenon. The level and duration of protein production depend on the amount of mRNA produced and its stability over time.

Interaction of plasmid/lipid complexes with the plasma membrane and intracellular trafficking of plasmids are still poorly understood. There are two leading hypotheses which describe the mechanism of cationic lipid-mediated transfection: (i) plasma membrane fusion and subsequent cytoplasmic delivery or (ii) a pathway involving endocytosis. These hypotheses are not mutually exclusive, and may be active to a greater or lesser extent in different cell types (45). Plasmid/lipopolyamine complexes are, for instance, reported to reach the cytoplasm after plasma membrane disruption along the endosomal route (7), possibly through osmotic swelling (44). Particles greater than 200 nm in diameter are not efficiently taken up by endocytosis and thus some large plasmid/lipid complexes may also be taken up by the cells via phagocytosis (46,47). Friend *et al.* (49) proposed, but did not prove, that plasmid/lipid complexes enter the cell by endocytosis via coated pit pathway. According to this proposal, plasmid/lipid complexes bind to cells, migrate to clathrin-coated pits of about 150 nm in diameter, leading to the formation of coated vesicles soon after pinching off from the cell surface and losing their clathrin coat. The transition from coated vesicle to early endosome is accompanied by acidification of the vesicular lumen that continues into the late endosomal and lysosomal compartments, reaching a final pH in the perinuclear lysosome of approximately 4.5.

Xu and Szoka (49) have recently proposed that plasmid/lipid complexes internalize into the endosome and initiate the destabilization of endosomal membrane. This destabilization induces flip-flop of cytoplasmic endogenous anionic lipids, which diffuse into the complexes and form charge neutralized ion pairs with the cationic lipids. This phenomenon displaces the plasmid from the complex and permits DNA entry into the cytoplasm. This proposal is partly based on the evidence that free plasmids injected into the nucleus will express whereas plasmid/lipid complexes injected in the nucleus will not (50,51). This suggests that plasmids need to be released from the plasmid/lipid complexes prior to entering the nucleus for expression to occur (51). Further investigation is required to confirm this proposal, as Gao and Huang (52) demonstrated that RNA polymerases are capable of displacing cationic lipids from DNA during transcription.

Even after being released from the complex into the cytoplasm, plasmid is still too large to enter the nucleus by simple diffusion, as the aqueous channel of the nuclear pore allows the free diffusion of only small particles (<~70 kDa). Yet

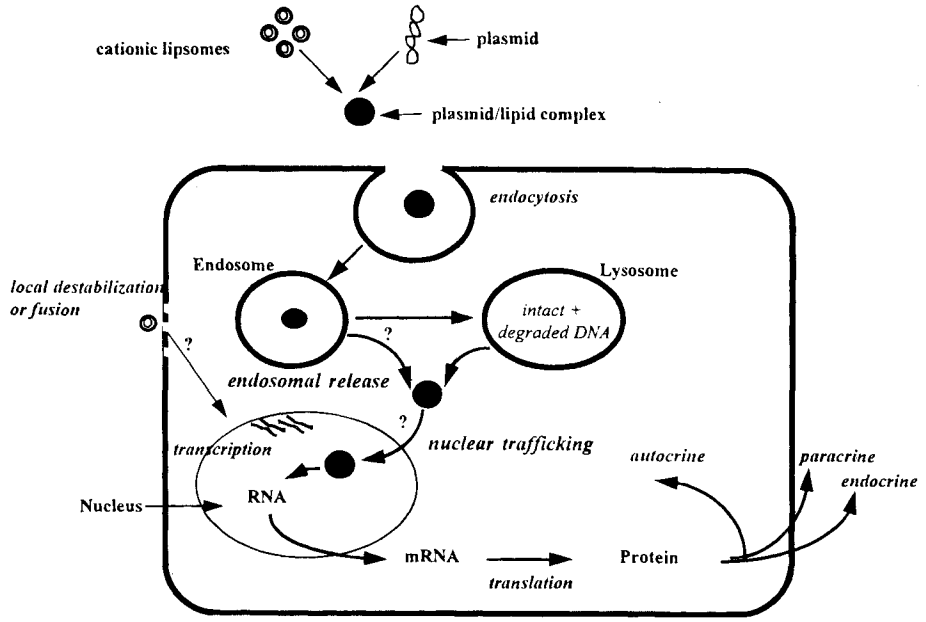

Fig. 2. Schematic representation of cationic lipid-mediated gene transfer. The pathway of gene transfer and expression involves delivery of plasmid/lipid complex to the cell surface, cellular uptake, endosomal release, transit from cytoplasm to nucleus, and expression of gene.

some plasmids clearly reach the nucleus, since gene expression is detected. Nuclear localization signal (NLS) is characterized by a short stretch of 5 to 10 basic amino acid residues present in some proteins and direct the localization of these proteins to the nucleus. It is believed that the basic region facilitates interaction with NLS binding proteins, which allow transport through nuclear pore complexes in an ATP-dependent manner (53). The well characterized NLS of simian virus 40 (SV40) large tumor antigen (T-antigen) promotes protein import into the nucleus. A synthetic peptide containing Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu residues of large tumor antigen sequence has shown to serve as a NLS when cross-linked to BSA or immunoglobulin G (54). Many other proteins have also been shown to harbor functional T-antigen like localization. Thus, inclusion of NLS in the plasmid/lipid complex formulation may facilitate the active transport of plasmids to the nucleus. Factors affecting delivery and expression of lipid-based gene delivery systems are summarized in Table 1.

CONCLUDING REMARKS

The goal of cationic lipid-mediated gene delivery systems is to increase the gene transfer efficiency of expression plasmids by controlling their physicochemical, pharmacokinetic and pharmacodynamic characteristics. A thorough understanding of intracellular delivery mechanism of plasmid/lipid complexes and the careful design of efficient lipid-based systems should lead to reduction in the overall amount of plasmid/lipid complexes needed to achieve a therapeutic level of gene expression. Research directed towards designing novel cationic lipids and formulations that are stable in plasma and other biological milieus are highly desirable. Plasmid-based gene expression systems, with suitable promoter/enhancer, intron, cDNA and other regulatory elements, should also be developed in parallel to further enhance the overall gene expression from plasmid/lipid complexes.

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