Gene Ther Mol Biol Vol 2, 59-68. August 7, 1998.

Cationic liposome-mediated transfection *in vivo* (review)

Dexi Liu* and Young K. Song

Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261

* Corresponding Author: Tel: (412) 648-8553; Fax: (412) 383-7436; E-mail: dliu@vms.cis.pitt.edu

Key words: gene therapy, cationic liposomes, gene delivery, non-viral vector

Summary

Cationic liposomes have been widely used as a transfection reagent to introduce gene into cells. Although much has been learned about the factors affecting transfection efficiency of liposomes *in vitro*, understanding on how efficient these lipid carriers are in delivering gene into cells *in vivo* is still lacking. Recent studies using reporter genes show that significant level of gene expression can be obtained in different organs including the lung, heart, spleen, liver and kidneys following an intravenous administration of DNA/liposome complexes into mice. In these studies, the cationic lipid to DNA ratio, structure of cationic lipids, liposome composition, and particle size of lipid particles were found to be important in determining the transfection efficiency of cationic liposomes. It was also found that gene expression from a single administration is transient but can be maintained by repeated administration. In this paper, we review the data that characterize the *in vivo* transfection mediated by systemically administered cationic liposome.

I. Introduction

Cationic liposomes have become a well established vehicle for introducing genes into cells. Many cationic lipids have been synthesized in the last decade and have been shown to be active in transfecting different type of cells (Behr *et al*., 1989; Felgner *et al*., 1987; Felgner *et al*., 1994; Gao and Huang, 1991; Hawley-Nelson *et al*., 1993; Lee *et al*., 1996; Leventis and Silvius, 1990; Rose *et al*., 1991; Solodin *et al*., 1995). In fact, cationic liposomes have become commercially available as transfection reagents and a few liposome formulations have been used in gene therapy clinical trials for treatment of cancer (Nabel *et al*., 1993; Nabel *et al*., 1994) and other genetic disorders such as cystic fibrosis (Caplen *et al*., 1995; Gill *et al*., 1997; Porteous *et al*., 1997; Sorscher *et al*., 1994).

Despite their commercial availability, wide use as gene carriers in preclinical and clinical experiments, the major success of cationic liposome-mediated gene transfer has been limited to *in vitro* cell culture systems and *in vivo* at restricted sites where a local regional administration can be applied. Therefore, efforts have been made in the past few years towards the development of an efficient lipid carrier for systemic gene delivery. The major advantage of systemic gene delivery over local injection is that many more sites and a greater number of cells in the body can be targeted. Thus, the successful development of lipid-based carriers for systemic transfection has a great potential for increasing the overall usefulness of lipid carriers in gene therapy. Using a CMV driven expression system containing cDNA of luciferase gene and commonly used cationic lipids, we have systematically examined the factors affecting the transfection efficiency of intravenously administered cationic liposomes in mice. In this article we summarize some of our findings concerning the physicochemical parameters affecting the transfection efficiency of cationic liposomes and the characteristics of transgene expression *in vivo*. We also present our view on the mechanisms involved in cationic liposome-mediated transfection.

II. Physicochemical parameters affecting the transfection activity of cationic liposomes

A. Cationic lipid to DNA ratio

The ratio of cationic lipid to DNA in DNA/liposome complexes has been shown to be one of the most important factors affecting the transfection efficiency. The optimal ratio

Figure 1. Effect of cationic lipid to DNA ratio on liposome transfection activity. DNA/liposome complexes were prepared by mixing different amount of DOTMA liposomes with plasmid DNA (pCMV-Luc) in PBS (see Song *et al*., 1997, for methods). Each mouse received 25 μg of pCMV-Luc plasmid DNA with different amount of DOTMA liposomes in 200 μl from tail vein. Luciferase activity was assayed 8 hours after iv injection in the lung (), spleen (), heart (), liver () and kidney (). Error bar represents SEM from three mice.

found for *in vitro* transfection is in the range of 3.6 to 9 (cationic lipid:DNA, nmol:μg) depending on the types of cationic liposomes, and cell types used (Zhang and Liu, unpublished data). However, for systemic transfection in a mouse model, a much higher cationic lipid to DNA ratio appears to be required for a better transfection into organs such as the lung, heart, liver, spleen and kidneys. As shown in **Figure 1**, while the level of gene expression in the lung is the highest among all internal organs examined, the level of gene expression in most of organs increases with increasing cationic lipid to DNA ratio. An optimal cationic lipid to DNA ratio for the lung is approximately 36 to 1 or greater under the experimental conditions.

Considering the fact that a large number of negatively charged molecules and cellular components exist in the blood, a higher cationic lipid to DNA ratio required for better transfection activity may indicate that the additional cationic liposomes are needed to promote the activity of DNA/liposome complexes. An additional possibility may be that the structure of DNA/liposome complexes formed at different cationic lipid to DNA ratios are different. For example, it is possible that cells under the artificial conditions of cell culture prefer the structural type of DNA/liposome complexes formed at a lower cationic lipid to DNA ratio, while cells *in vivo* are more sensitive to the complex structure formed at a higher cationic lipid to DNA ratio. To test these possibilities, different amounts of free liposomes were injected intravenously into mice prior to the injection of DNA/lipid complexes prepared at low cationic lipid to DNA ratio (6:1= nmol:μg). **Figure 2** shows that, except for the spleen, the level of luciferase activity in all examined organs increased with increasing amounts of free liposomes pre-injected. The pattern and level of gene expression in different organs are very similar to those shown in **Figure 1**. These results suggest that free liposomes enhance the transfection efficiency of DNA/liposome complexes *in vivo*. The structures of DNA/liposome complexes formed at either low ratio (6:1) or high ratio (36:1) of cationic lipid to DNA are equally active. Therefore, free liposomes play an important role in determining the level of transgene expression following a systemic administration of DNA/liposome complexes.

B. Structure of cationic lipids

Since the first report on cationic liposome-mediated transfection by Felgner and his colleagues (Felgner *et al*., 1987), many new cationic lipids have been synthesized and shown to be effective in transfecting cells *in vitro* (for review, see Gao and Huang, 1995). To test whether lipid structure also plays an important role in the transfection of cells, liposomes were prepared using different types of cationic lipids. For all transfections summarized in **Figure 3**, DNA/liposome complexes were prepared at a cationic

Figure 2. Dose dependent effects of pre-injected free liposomes on the level of gene expression. DNA/liposome complexes were prepared at DOTMA to DNA ratio of 6:1 (nmol:μg) with lipid formulation of DOTMA/Tween 80 (6:2, weight ratio).Twenty five μg of pCMV-Luc plasmid complexed with DOTMA/Tween 80 formulation were injected 1 minute via tail vein after the animals received different amounts of DOTMA liposomes without Tween 80. Luciferase activity in different tissues was assayed 8 hours after the injection of DNA/lipid complexes. Results represent mean ± SEM of values obtained from 3-6 mice. Lung (), spleen (), heart (), liver () and kidney ().

Figure 3. Effect of cationic lipid structure on transfection activity of liposome-mediated transfection. Luciferase activity was assayed 20 hours after iv injection of 25 μg of pCMV-Luc plasmid complexed with different cationic liposomes in the lung (), spleen (), heart (), liver () and kidney (). Cationic lipid to DNA ratio used was 36:1 (nmol:μg). Error bar represents SEM from three mice.

Figure 4. Size effect on liposome-mediated transfection. Liposomes composed of either DOTMA or DOTAP with different liposome diameter were complexed with pCMV-Luc plasmid DNA at a cationic lipid to DNA ratio of 36:1 (nmol:μg). The size of liposomes and DNA/liposome complexes represents an average size of particles measured by laser light scattering. Luciferase activity was determined 20 hours post-injection in the lung (), spleen (), heart (), liver () and kidney (). Error bar represents SEM from three mice.

lipid to DNA ratio of 36:1. It is evident from **Figure 3** that the transfection activity of cationic liposomes varies significantly with cationic lipid structure. Between the two types of cationic lipid tested, 3[N-(N', N'-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol), a cholesterol derivative with a tertiary amine as the charged head group (Gao and Huang, 1991), exhibited a low transfection activity in comparison to alkyl chain-based lipids such as N-(2,3-dioleoyloxy)propyl-N,N,N-trimethylammonium chloride (DOTMA) (Felgner *et al*., 1987) and 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP) (Leventis and Silvius, 1990). Compared to DOTAP liposomes, the level of gene expression in the lung of animals transfected with DOTMA liposomes is approximately 10-fold higher. As the only structural difference between DOTMA and DOTAP is the linkage between the hydrophilic head group and the alkyl chains, these results suggest that the ether linkage (DOTMA) between the head group and the alkyl chains is superior to the ester linkage (DOTAP). In addition, **Figure 3** also shows that the structure of hydrophobic portion of the lipid molecule is also important for the ultimate transfection activity. Liposomes composed of lipids with shorter [1,2-dimyristoyl-3-trimethylammonium propane (DMTAP), C_{14})] or longer but with unsaturated alkyl chains (DOTAP, $C_{18:1}$) exhibit better activity than those with long and saturated alkyl chains [1,2-dipalmitoyl-3-trimethylammonium propane (DPTAP), C_{16} and 1,2-distearoyl-3-trimethylammonium propane (DSTAP), C_{18}]. Among the cationic lipids tested, DOTMA liposomes appear to be most active under our experimental conditions.

C. Diameter of lipid particles

The particle diameter of liposomes and DNA/liposome complexes is another parameter that was examined for its effect on transfection activity of cationic liposomes. It is apparent in **Figure 5** that the diameter of DNA/liposome complexes is directly related to the liposome diameter. For example, the average diameter of DNA/liposome complexes was around 400-500 nm when small sized liposomes (87 nm) were used, compared to a diameter of more than 1 μm when the average liposome diameter was around 750 nm.

Figure 5. Effect of neutral lipid on the transfection activity of cationic liposomes. Each mouse received 25 μg of pCMV-Luc plasmid complexed with different liposome composition at cationic lipid to DNA ratio of 36:1 (nmol:μg). The cationic lipid to neutral lipid ratio in liposomes was 1:1 (molar ratio). Luciferase activity was determined 8 hours post-injection in the lung (), spleen (), heart (), liver () and kidney (). Error bar represents SEM from three mice.

This pattern did not seem to depend on the structure of cationic lipids since similar increases in particle size of DNA/liposome complexes were obtained for both DOTMA and DOTAP liposomes. Interestingly, transfection activity of DOTAP liposomes appears to depend on liposome size. An approximately 10-fold increase of luciferase activity in the lung was seen when the size of DOTAP liposomes increased from below 100 to 450 nm or greater. Such size dependence was unique to DOTAP liposomes and was not observed with DOTMA liposomes.

D. Effect of "helper" lipids

Inclusion of a neutral lipid such as dioleoylphosphatidyl-ethanolamine (DOPE) into the cationic liposomes has been a common practice in cationic liposome-mediated transfection (Behr *et al*., 1989; Felgner *et al*., 1987; Felgner *et al*., 1994; Gao and Huang, 1991; Hawley-Nelson *et al*., 1993; Lee *et al*., 1996; Leventis and Silvius, 1990; Rose *et al*., 1991; Solodin *et al*., 1995). In fact, most commercially available cationic liposomes contain DOPE (Gao and Huang, 1995). It is generally believed that, once inside a cell, DOPE in DNA/liposome complexes can facilitate the transfer of DNA across the endosomal membrane and thereby, enhance transfection activity (Farhood *et al*., 1995; Legendre and Szoka, 1992; Wrobel and Collins, 1995). In addition, several studies have also shown that inclusion of cholesterol into cationic liposomes can enhance the transfection activity at lower cationic lipid to DNA ratios (Bennett *et al*., 1995; Hong *et al*., 1997; Y. Liu *et al*., 1997; Templeton *et al*., 1997). While it appeared to be true that transfection activity of DOPE-containing liposomes is better than liposomes made of cationic lipid alone under simplified *in vitro* conditions, it was not clear, however, whether this would hold true *in vivo* when an optimal cationic liposome to DNA ratio was used. To test whether DOPE and cholesterol have a positive effect on the transfection activity of cationic liposomes *in vivo*, liposomes containing equal amounts of cationic lipid and DOPE or cholesterol were prepared. Their *in vivo* transfection activity was then tested in mice using a standard protocol. **Figure 5** shows that inclusion of DOPE or cholesterol into DOTMA or DOTAP liposomes does not seem to further enhance the level of gene expression when the cationic lipid to DNA ratio was optimal (36:1, nmol:μg). Furthermore, these results suggest that, despite the fact that DOPE was previously shown to be effective in enhancing the transfection activity of liposomes *in vitro*, it may not be efficacious to include DOPE as a "helper lipid" into liposomes prepared for systemic gene delivery. These results also indicate that the composition of DNA/liposome complexes optimized under one condition may not be optimal in a different condition. Hence, the optimal composition of the lipid systems for transfection is likely to be condition dependent.

Figure 6. Dose response curve. Various amounts of pCMV-Luc plasmid DNA complexed with DOTMA liposomes at a lipid to DNA ratio of 36:1 (nmol:μg) were intravenously injected into mouse and luciferase activity was determined 20 hours post-injection in the lung (), spleen (), heart (), liver () and kidney (). Error bar represents SEM from three mice.

Figure 7. Time dependent gene expression in different tissues. Luciferase activity in the lung (), spleen (), heart (), liver () and kidney () was assayed at various time point in animals each receiving 25 μg of pCMV-Luc plasmid complexed with DOTMA liposomes (cationic lipid:DNA=36:1, nmol:μg). Error bar represents SEM from three mice.

E. Dose response curve

The dose response curve for DOTMA liposomes is shown in **Figure 6**. It is clear that the level of luciferase activity increased as the injected dose was increased. The highest increase in luciferase activity was obtained in the lung. For example, an approximately 10- to 100-fold increase in luciferase activity was seen in the lung when the injected DNA dose was increased from 10 to 75 μg/mouse. Under these conditions, the level of luciferase activity appeared to be saturated at 50 μg DNA/mouse.

III. Characteristics of transgene expression

A. Time dependent gene expression

Expression of the transgene in the tissues is transient. The results in **Figure 7** show that gene product can be detected as early as 2 hours, reaches the maximal level in the lung around 8-10 hours and decreases to less than 1% of the peak level in 4 days. Among the organs tested, including the lung, spleen, liver, heart and kidney, the level of gene expression in the liver was the most transient. It reaches its highest level of 10^5 relative light units per mg extracted proteins (RLU/mg) 2-3 hours post injection and dropped to a minimal level in about 48 hours, suggesting that liver may have a higher degradation rate for gene or/and gene product.

The relationship between the level of gene expression and the amount of transgene introduced into the lung as a function of time was established using Southern analysis. In these experiments, animals were sacrificed at 4, 12, 24, 48, 72, 120 and 168 hours after DNA/liposome complexes were injected. A DNA extract from the lungs was prepared and the relative level of transgene in each sample was analyzed using ^{32}P-labeled full length luciferase gene as a probe. As shown in **Figure 8**, the amount of luciferase gene detected in the lung decreased with time. Five days after injection, the level of luciferase gene in the lung was below the detectable level for our experimental conditions. These results indicate that the transient gene expression is likely due to the instability of the transgene in transfected cells.

B. Effect of repeated injection

While it is expected to observe a transient gene expression in cationic liposome-mediated transfection, it is important to demonstrate whether the level of gene expression can be maintained by repeated administration. In fact, sustaining the level of gene expression by repeated administration is considered as one of the most attractive features for nonviral gene delivery systems. The results in **Figure 9** provide direct support to such a prediction. It is evident that a similar level of gene expression in all internal organs was obtained by a repeated administration of DNA/liposome complexes. Interestingly, however, a high level of gene expression may not be achieved if the second injection was performed before approximately

14 days after the first injection of DNA/liposome complexes. A period of about two weeks or more between the two injections is needed for an optimal transfection from the second administration.

IV. Discussion

It is clear from our work (F. Liu *et al*., 1997; Song *et al*., 1997; Song and Liu, in press) and the work of others (Hong *et al*., 1997; Li and Huang; 1997; Y. Liu *et al*., 1997; McLean *et al*., 1997; Templeton *et al*., 1997; Thierry *et al*., 1995; Zhu *et al*., 1993) that cationic liposomes are indeed effective in transfecting cells *in vivo* by systemic administration of DNA/liposome complexes. Although gene product can be detected in many different organs (lung, spleen, heart, liver and kidneys), the highest level was found in the lung. Such a high level of gene expression in lung is likely due to the fact that pulmonary vasculature is the first capillary bed encountered by the DNA/liposome complexes after intravenous injection. DNA/liposome complexes, once injected into the blood stream via the tail vein, may bind to the endothelial cells lining the capillary bed of the blood vessels in the lung. The embolic effect, resulting from the interaction of DNA/liposome complexes with blood components, and with the negatively charged surface of the endothelial cells of the blood vessel, presumably plays a major role in generating a high level of gene expression in lung.

Figure 9. Effect of repeated injection on the level of gene expression in different tissues. Twenty five μg of pCMV-Luc plasmid complexed with DOTMA liposomes at lipid to DNA ratio of 36:1 (nmol:μg) were injected intravenously on days 4, 7, 14, 19 and 23 respectively, into mice that had received the same dose and type of DNA/liposome complexes on day zero. The luciferase activity in the lung (), spleen (), heart (), liver () and kidney () was assayed 20 hours after the second injection. In control group (con), mice received only one injection. Error bar represents SEM from three mice. The dose of plasmid DNA injected in this experiment was adjusted to 1.25 mg/Kg based on animal weight.

lung capillary bed. A slower flow would lengthen the exposure time of DNA to the endothelial cells lining the vascular wall and result in a higher level of gene expression. Therefore, DNA/liposome complexes that are capable of being trapped in the lung for an extended period of time will produce a successful transfection. Although many biological factors may be involved, different physicochemical parameters such as cationic lipid to DNA ratio, cationic lipid structure, diameter of the lipid particle and inclusion of helper lipids (**Figure 5**) may all affect the DNA retention time with the cells to be transfected. It is possible that the DNA retention time with cells, before and after gene transfer occurs, ultimately determines the level of gene expression. Results from biodistribution studies with ^{125}I-labeled plasmid DNA appear to support this hypothesis (F. Liu *et al*., 1997; Song and Liu, in press).

The suppression effect is caused by DNA/liposome complexes but not by free liposomes, the plasmid DNA or gene product (D. Liu, 1997).In summary, the results presented in this paper and those published by other laboratories (Hong *et al*., 1997; Li and Huang; 1997; Y. Liu *et al*., 1997; McLean *et al*., 1997; Templeton *et al*., 1997; Thierry *et al*., 1995; Zhu *et al*., 1993) suggest that the lung is the most transfectable organ by cationic liposomes through intravenous administration. While this may offer an advantage for delivering genes to lung endothelial cells, it also provides a barrier for delivering genes to cells in other organs. Further studies will be required to define the mechanisms by which cationic liposomes or/and complexes interact with cells in the presence of blood as well as the effect of dynamics of blood flow. Studies are also needed to provide information on how plasmid DNA is transferred to various intracellular compartments of cells in different organs. A better understanding of the nature of these processes, and of how gene transfer efficiency is influenced by multiple physicochemical parameters may allow the development of new strategies for further improvement.

Acknowledgment

This work was supported in part by a grant from National Institute of Health CA 72925 and by Targeted Genetics Corporation.

Received 19 May 1998; accepted 3 June1998

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