In Vitro Myotoxicity of Selected Cationic Macromolecules Used in Non-Viral Gene Delivery

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Purpose. Cationic lipid/DNA complexes have been proposed as a method of *in vivo* gene delivery via intravenous or intramuscular injection. A concern with using these polycationic molecules is whether they are associated with tissue toxicity at the injection site. Therefore, the objective of these studies was to investigate the myotoxic potential of selected non-viral gene delivery macromolecules (e.g., cationic lipids and polymers) with and without plasmid DNA (pDNA) *in vitro*.

Methods. Myotoxicity was assessed by the cumulative release of creatine kinase (CK) over 90 minutes from the isolated rodent extensor digitorum longus muscle into a carbogenated balanced salt solution (BBS, pH 7.4, 37°C) following a 15 μL injection of the test formulation. Phenytoin (Dilantin®) and normal saline served as positive and negative controls, respectively.

Results. The myotoxicity of plasmid DNA (pDNA, ~5000bp, 1 mg/ml) was not statistically different from normal saline. However, the myotoxicity of Dilantin® was 16-times higher than either normal saline or pDNA ($p < 0.05$). Cationic liposomes were found to be less myotoxic than polylysine and PAMAM dendrimers. Polylysine's myotoxicity was found to be dependent upon concentration and molecular weight. The myotoxicity of formulations of cationic liposomes(s), lower molecular weight polylysine (25,000) and higher concentration of PAMAM dendrimers with pDNA were found to be statistically less significant than those formulations without pDNA.

Conclusions. The cationic liposomes were less myotoxic compared to the dendrimers and polylysine. Myotoxicity was dependent upon the type of cationic lipid macromolecule, concentration, molecular weight and the presence of pDNA. A possible explanation for this reduced tissue damage in cationic lipids complexed with pDNA is that the formation of complex reduces the overall positive charge of the injectable system resulting in less damage.

KEY WORDS: myotoxicity; plasmid DNA; liposomes; dendrimers; polylysine; gene delivery.

INTRODUCTION

The parenteral administration of naked or plasmid DNA (pDNA) for gene therapy has been suggested for the treatment of a variety of disorders including cancer, cystic fibrosis and muscular dystrophy (1–3). Effective use of pDNA as a drug will require delivery systems due to metabolic instability and barriers to cellular uptake. Several non-viral adjuvants (e.g., cationic liposomes, polylysine, and dendrimers) are currently in development for the effective production of transgene drugs using both *in vivo* and *in vitro* models.

A key consideration in the development of these formulations containing cationic liposomes, polylysine or dendrimers for parenteral administration will be the toxicity of the various pDNA delivery systems. Since these molecules are positively charged and can be considered a type of polycation, the toxicity of such pDNA delivery systems might arise from a direct interaction of the formulation components with the external or internal cellular membranes at the site of injection. It has been shown that polycations, which bind to the negatively charged surface of mammalian cell membranes, can cause charge neutralization, cell distortion, lysis and agglutination (4–5). Furthermore, polycations can also bind to cell nuclei and internal membranes and interfere with enzyme and cellular function (6–7). Alternatively, the toxicity of these molecules could be a consequence of immune system stimulation (8).

The isolated rodent model is an established system for the evaluation of myotoxicity (e.g., muscle damage) following the direct injection into an isolated muscle (9–10). This system, which uses the release of the cytosolic enzyme creatine kinase (CK), can be used to rapidly screen individual formulation components and complete formulations for their potential to cause acute damage at an intramuscular injection site. The purpose of the present study was to investigate the myotoxicity of several different classes of macromolecules (e.g., cationic liposomes, dendrimers and polylysine) that may be used for non-viral gene delivery. We also examined the myotoxicity of these polycations when they were complexed with pDNA at different concentrations. It can be postulated that the addition of a negatively charged pDNA molecule would reduce the myotoxicity of these cationic molecules by a reduction in the overall positive charge of the cationic molecule-pDNA complex.

MATERIALS AND METHODS

Materials

The structures of the cationic molecules used in this study are shown in Figure 1. The cationic liposomes were combinations of the lipids 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), dioleoyl phosphatidylethanolamine (DOPE) and 3[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and DC-Chol was obtained from Sigma Chemical Company (St. Louis, MO). Liposomes were prepared by mixing lipids in chloroform and evaporating the solvent with a Buchi Rotavapor at 60°C. The dried lipid film was reconstituted in sterile water with shaking as described previously (11). These liposomes were evaluated at concentration of 1 and 5 mg/ml. The concentration is in terms of only the cationic lipid. In the mixed liposome formulations, the ratio of DC-Chol:DOPE AND DOTAP:DOPE was 1:1 (mol:mol).

Polylysine (25 and 37KD) was bought from Sigma Chemical Company and dissolved in normal saline. Generation 4 dendrimers were purchased from Aldrich Chemicals (St. Louis, MO). The dendrimers were supplied in methanol which was removed by evaporation and replaced with phosphate buffered saline. Polylysine (dl form, 37KD) and PAMAM dendrimers

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ABBREVIATIONS AND ACRONYMS: DOTAP, 1,2-dioleoyl-3-trimethylammonium propane; DOPE, dioleoyl phosphatidylethanolamine; DC-Chol, 3[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol; CK, creatine kinase; pDNA, plasmid DNA; EDL, extensor digitorum longus.

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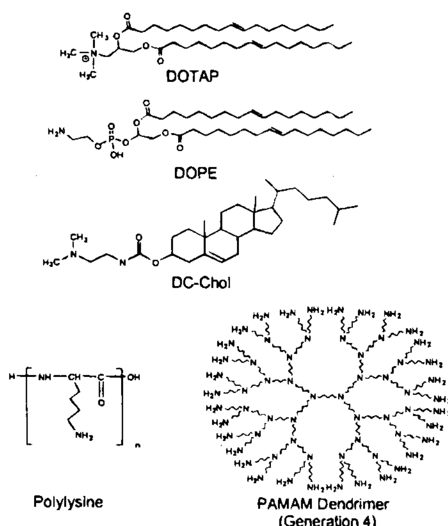

Fig. 1. Representative structures of cationic lipids, polylysine and PAMAM dendrimers.

(Generation 4) were investigated at 1 and 5 mg/ml. A 1 mg/ml solution of the 25 KD polylysine was used in some studies.

pDNA (pGL3 luciferase firefly plasmid with SV-40 promoters and enhancers) was obtained from Promega and transfected into and isolated from *Eschericia coli,* strain JM-109. Plasmids were purified using a Wizard MegaPrep kit (Promega, Madison, WI). DNA was stored in TE buffer pH 7.0 at a concentration of 1 mg/ml. The concentration and purity of pDNA were determined spectrophotometrically (12). The mixtures of pDNA and cationic molecules were prepared as a weight to weight ratio prior to the injection procedure. The mixtures are represented as the weight of the cationic lipid and the weight of the pDNA. Phenytoin (Dilantin®) and normal saline, the positive and negative controls, are manufactured by Parke-Davis and Abbott Laboratories, respectively. All other chemicals used in these studies were of the highest commercial purity or grade.

In Vitro Myotoxicity Studies

Extensor digitorum longus (EDL) rodent muscles (approximately 200 mg) were isolated from male Sprague Dawley rats as previously described (9–10). Briefly, rodents were administered an anesthetic dose of pentobarbital and sacrificed via cervical dislocation as approved by the Animal Care and Use Committee at the University of Florida in accordance with the National Institutes of Health Guidelines. The EDL muscles were injected with the test solution (15 μL) using a 100 μL Hamilton syringe equipped with a needle guard to control the depth and angle of injection. The injected muscles were placed into a teflon coated plastic basket and immersed in 9 ml of carbogenated (95% O_2/5% CO_2) balanced salt solution (BSS). The solutions were drained and fresh BBS added at 30-minute intervals. These drained solutions at 30, 60 and 90 minutes were analyzed for creatine kinase (CK) using a commercially available spectrophotometric kit (Sigma Chemical Company, No. 47, St. Louis, MO) at 340 nm and a Cary 3E UV-Visible spectrophotometer. Myotoxicity is calculated from the sum of the creatine kinase values determined in the 30, 60 and 90 minute samples. This value isexpressed as the cumulative release of creatine kinase (U/L) over the 90 minute period as described in early studies using this model (9–10).

Statistics

Data are presented as the mean and standard error of the mean with 3 to 4 muscles per treatment. All analyses were performed using analysis of variance (ANOVA) and the Fisher's post-hoc test with $p < 0.05$ considered statistically significant. The differences between the positive control - Dilantin®, negative control-normal saline and pDNA was analyzed using analysis of variance (ANOVA) and the Fisher's post-hoc test with $p < 0.05$ considered statistically significant.

RESULTS

The isolated muscle system has been shown to retain muscle viability for approximately 120 minutes (10). Therefore, to eliminate the possibility that decreased muscle viability contributed to increased release of creatine kinase, myotoxicity of the formulations was determined at 90 minutes post-injection. The cumulative myotoxicity of the negative (normal saline) and positive (Dilantin®) controls as well as pDNA injected alone are shown in Figure 2. There was no statistical difference between the myotoxicity of normal saline versus pDNA. However, the myotoxicity of Dilantin® was 16-times higher ($p < 0.05$) compared to either normal saline or pDNA. The amount of pDNA injected was 15 μg, while the amount of phenytoin was 750 μg.

A comparison of the three cationic classes (e.g, liposomes, proteins, and dendrimers) of molecules suggests cationic lipids cause the least myotoxicity, while dendrimers (generation 4) were associated with the highest myotoxicity (Figures 3–5). The myotoxicity of cationic liposomes with or without pDNA are shown in Figure 3. DC-Chol:DOPE liposomes were found to be statistically less myotoxic than DOTAP (1 mg/ml) or DOTAP/DOPE (at 1 or 5 mg/ml) liposomes. The myotoxicity of DC-Chol:DOPE was statistically lower than Dilantin®, but it was not different from normal saline or pDNA. In contrast, the myotoxicity of DOTAP liposomes (1 mg/ml) and DOTAP/DOPE liposomes (1 or 5 mg/ml) were found to be statistically significant compared to normal saline or pDNA, while at the same time being statistically smaller than that caused by Dilantin®. For DOTAP:DOPE liposomes, the myotoxicity was concentration-independent with no statistically significant difference between the low (1 mg/ml) and the higher concentra-

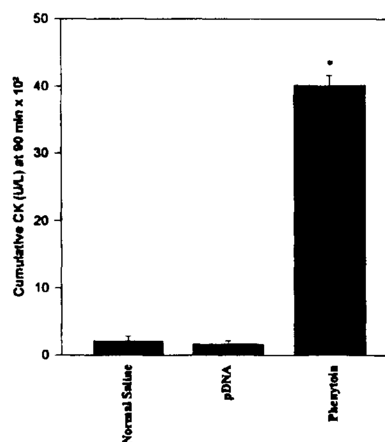

Fig. 2. Myotoxicity of pDNA compared to normal saline (negative control) and Dilantin® (positive control). Symbols: * - statistically significant from normal saline and pDNA ($p < 0.05$).

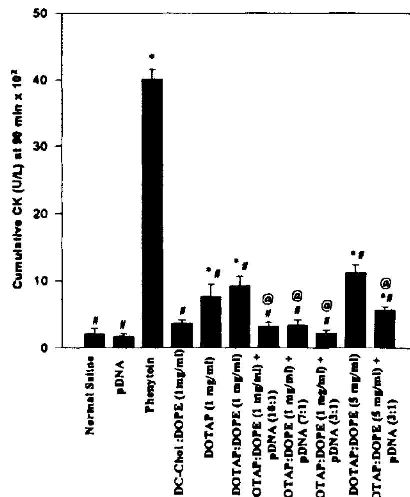

Fig. 3. Myotoxicity of selected cationic liposome treatments with or without pDNA. The values of normal saline, pDNA and Dilantin® are the same as in Figure 2 and are provided to allow comparison with these treatments. Symbols indicate statistical significance ($p < 0.05$): # - Different from Dilantin®, * - Different from normal saline and pDNA, and @ - Different from liposome formulation without pDNA.

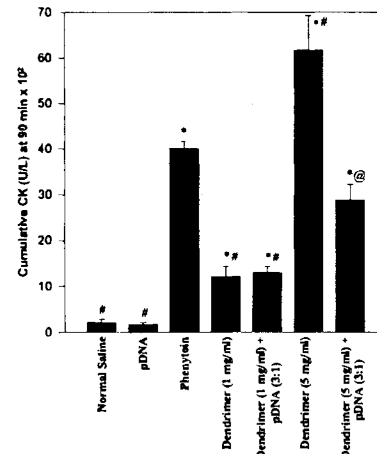

Fig. 5. Myotoxicity of selected dendrimer treatments with or without pDNA. The values of normal saline, pDNA and Dilantin® are the same as in Figure 2 and are provided to allow comparison with these treatments. Symbols indicate statistical significance ($p < 0.05$): Symbols indicate statistical significance ($p < 0.05$): # - Different from Dilantin®, * - Different from normal saline and pDNA, and @ - Different from dendrimer formulation without pDNA.

tion (5 mg/ml). The addition of pDNA to DOTAP:DOPE liposomes resulted in a statistically significant reduction in myotoxicity compared to the liposomes without the pDNA.

The cumulative myotoxicity of the polylysine molecules with or without pDNA is shown in Figure 4. There appears to be both a dose and a molecular weight component to the myotoxicity of this cationic macromolecule. Polylysine 37,000 (1 and 5 mg/ml) and polylysine 25,000 (5 mg/ml) were statistically more myotoxic than normal saline or pDNA. Likewise, polylysine 37,000 (1 mg/ml) and 25,000 (5 mg/ml) were statistically less myotoxic than Dilantin®. In contrast, polylysine 37,000 (5 mg/ml) was not statistically different from Dilantin®. The polylysine 37,000 was statistically more myotoxic than the polylysine 25,000 at the same concentration (5mg/ml). Myotoxicity of the polylysine 37,000 was concentration-dependent with the higher concentration approximately two-times greater than the lower concentration. The addition of pDNA did not result in a statistically significant reduction in myotoxicity of the polylysine 37,000 at 1 or 5 mg/ml. However, the addition of pDNA to the polylysine 25,000 did statistically reduce the extent of myotoxicity by approximately two-fold.

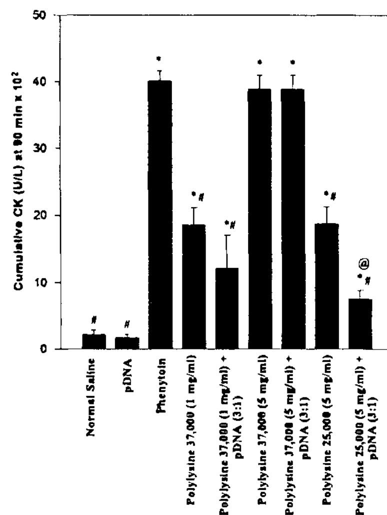

Fig. 4. Myotoxicity of selected polylysine treatments with or without pDNA. The values of normal saline, pDNA and Dilantin® are the same as in Figure 2 and are provided to allow comparison with these treatments. Symbols indicate statistical significance ($p < 0.05$): Symbols indicate statistical significance ($p < 0.05$): # - Different from Dilantin®, * - Different from normal saline and pDNA, and @ - Different from polylysine formulation without pDNA.

The myotoxicity of the PAMAM dendrimer (Generation 4) is shown in Figure 5. The dendrimer (1 mg/ml) was not statistically different from normal saline, but was statistically more myotoxic than pDNA and less myotoxic than phenytoin. The higher dendrimer (5 mg/ml) concentration caused statistically more myotoxicity than normal saline, pDNA and Dilantin®. The addition of pDNA did not reduce the myotoxicity of the 1 mg/ml dendrimer, however when plasmid DNA was mixed the higher dendrimer concentration (5 mg/ml) the myotoxicity was significantly reduced by three-fold compared to the dendrimer alone.

DISCUSSION

Non-viral gene delivery may be an important new therapeutic option in the future. The use of non-viral systems has several advantages over viral delivery of DNA in that the investigator has control over the design and construction of the delivery vector (13). In order to accomplish the successful delivery of DNA (e.g., plasmid DNA), a better understanding of the delivery vector is required. Studies have recently revealed important aspects of the delivery in regards to specific lipids (14), methods of formulation (15), and targeting systems (16) increasing the general understanding of the versatility of the delivery systems. Successful expression of a transgene requires additional information on tissue toxicity in acute and chronic animal models. A well-studied model for

addressing acute toxicity or tissue damage following an intramuscular injection is the isolated rodent skeletal muscle model (9–10). The advantage of this experimental system is it permits the investigator to rapidly screen treatments or formulations for their potential to cause acute tissue damage and to compare the extent of this toxicity to previously established non-toxic and myotoxic formulations. Furthermore, this system can be modified to examine whether the toxic effect of the tested compound or formulation is a consequence of a direct effect on the cellular membrane or secondary to other toxic effects on intracellular biochemical pathways (17). The limitation with this system is that it does not allow the determination of toxic effects following repeated injections or the administration of long-acting injectables that may elicit an immune response. These types of studies are best conducted using the *in vivo* rodent or rabbit model of muscle damage.

The injection of pDNA does not cause acute muscle damage in our model system nor has damage been reported in other studies addressing transgene expression (18–19). The total amount of plasmid DNA used in these studies was 15 μg. The amounts of plasmid used in animal studies have varied from nanograms (for immune stimulation) (20) up to 200 μg for expression of reporter genes (21) in muscles. In these studies the dose chosen was not meant to mirror doses which are to be used in clinical studies but do address possible toxicity concerns with the administration of DNA and its delivery vectors. While it may seem that the total amount of cationic molecule with or without pDNA injected into these muscles is small (viz., 15 μl of the test solution injected into a muscle weighing approximately 200 mg), this injection volume has been shown to easily discriminate between those non-toxic and toxic compounds as demonstrated by the 16–20 fold difference between normal saline (negative control) and Dilantin® (positive control) reported in the present and previous studies (23). Molecules other than pDNA such as endotoxin and DNAase may contribute to damage caused by non-viral gene delivery systems if the plasmids are not properly isolated (22–25).

The concentrations of the cationic molecules chosen for the studies were based on formulations currently commercially obtainable such as Lipofectin (Gibco BRL), a 1 mg/ml cationic lipid and other concentrations, which have been used in the literature (13). One mg per ml of the cationic liposome is the most commonly reported and it is reportedly used in ratios from 1:2 w/w (DNA:liposomes) up to 1:32 or greater. An excess of positive charge is reported to be necessary for successful transfection of cells (26). If we assume the average molecular weight from many of the cationic lipids is around 750 g/mol then charge neutrality would occur at ratio of approximately 1:3 w/w (DNA/liposomes) for the 15 μg of DNA used in the current study along with the permanently charged lipid (DOTAP). Based on this calculation a formulation near neutrality and one with an overall positive charge were evaluated. Charge neutrality would occur at different ratios for the amines which are titratable (DC-Chol, poly-lysine, and dendrimers) depending on the local pH microenvironment. In the present study the gold standard of non-viral transfection systems was assumed to be the permanently charged cationic lipid.

DOTAP:DOPE, polylysine and dendrimers caused more acute muscle damage than normal saline or the injection of pDNA itself. Cationic compounds (e.g., cationic surfactants) have been reported to be toxic in a variety of tissue culture systems (4–8,27). Interestingly, the combination of cationic vectors and anionic pDNA resulted in a decrease in muscle damage. Possible mechanisms to explain the reduction in acute myotoxicity of cationic molecules by the addition of DNA are 1) the formation of complexes which reduce the overall positive charge of the injectable systems, 2) changes in particle size, and 3) the potential free radical scavenger properties of pDNA. The sizes of cationic molecules and plasmid DNA have been reported in the literature. Sizes of cationic lipid:DNA complexes are reported to be spherical or lipid coated strands of nucleic acid in the 100–200 nm range, while polylysine complexes are reported to be doughnut shaped between 70–100 nm (28). It is clear that the initial DNA/cationic molecule size populations are extremely heterogeneous and that the size will change with time and the environment to which it is introduced into during a particular study.

It appears that there is a difference in the ability of cationic molecules to cause acute muscle damage. The differences in toxicity may lead to important alterations of transgene expression. From the liposome studies (Figure 3), it appears the cationic DC-Chol:DOPE lipid (a tertiary amine) was less toxic than the quaternary nitrogen in DOTAP, although structural considerations may also influence the relative toxicity of the molecules. The addition of pDNA to the cationic liposomes resulted in a reduction in the total cumulative release of creatine kinase. The decrease in toxicity could arise from changes in the shape or size of the cationic liposomes, or the reduction of total number of cationic particles due to condensation with the plasmid. In addition, DNA has been reported to be a free radical scavenger, but this effect was unlikely considering in the model system the addition of pDNA to Dilantin® treatment did not alter the toxicity (data not shown).

There also appears to be a direct relationship between molecular weight of the polylysine and tissue damage measured by the cumulative release of creatine kinase. Several molecular weight polylysines have been demonstrated to have activity in the literature; the range has been from the low 20,000 up to 70,000. The larger molecular weight polymer might have increased toxicity due to its slower diffusion from the injection site or decreased metabolism or degradation in muscle. Figures 4 and 5 also demonstrate the ability of pDNA to reduce myotoxicity for selected polylysine and dendrimer formulations. The reason for the selective protection, which appears to be concentration and/or molecular weight dependent, is currently not known.

In comparing the three cationic categories with regards to the total cumulative creatine kinase released, liposomes < polylysine < dendrimers. The differences in toxicity may be due to structural differences in these molecules. In previous studies, empty liposomes composed of egg-phosphatidylcholine, egg-phosphatidylglycerol, cholesterol and stearylamine did not appear to be myotoxic. Furthermore, liposomal size, charge and fluidity did not affect the myotoxicity of these selected liposomes (20). Other factors that could affect the myotoxicity of these cationic molecules include the presence of the positive charge within or outside the molecule or the ability of the biological system to clear the molecule from the injection site. Theoretically, in the liposome only treatment a portion of the cationic charge is within the liposome, which may alter the creatine kinase release compared to polylysine and dendrimers where the entire positive charge may have access to the tissue at the injection site. Furthermore, it is possible that polylysine and dendrimers are less likely to be degraded in the test system.

Finally, it can be speculated that the magnitude of the positive charge of the formulation (with or without pDNA)

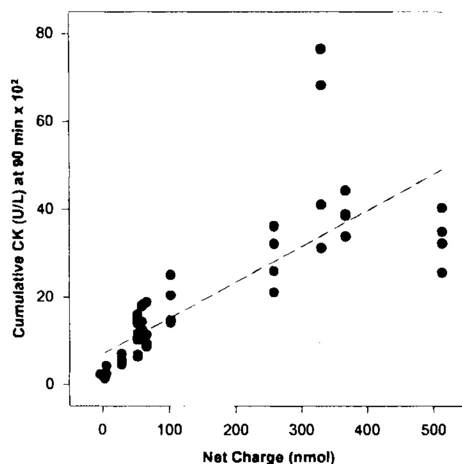

Fig. 6. Relationship between net charge of the complex and creatine kinase released. Each point represents a separate experiment. The titratable amines (lysines and denrimers) were assumed to protonated in the calculations. The r^2 value for the fit was 0.63.

could be related to the toxicity of the molecule. In Figure 6 we have graphed the net charge of the particle vs. the creatine kinase released. The figure indicates that there is a relationship between these two parameters with a r^2 value of 0.63. The two outliers in the data set were from the higher dendrimer concentration. It becomes critical to determine the net-positive charge of the molecules. There may be a correlation between cumulative creatine kinase release and the overall net-positive charge of the pDNA:polycation complex. This speculation is interesting since in many tissue culture models the ratio of cationic molecules to plasmid DNA is often greater than that required for *in vivo* studies.

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