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Nonadditive Effects of Repetitive Administration of Lipoplexes in Immunocompetent Mice

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ABSTRACT

Repetitive administration is routinely used to maintain therapeutic drug levels, but previous studies have documented an accelerated blood clearance of some lipid-based delivery systems under these conditions. To assess the effect of repetitive administration, non-PEGylated lipoplexes (+/−0.5) were administered 4 times via tail vein injection at 3-day intervals to immunocompetent BALB/c mice bearing 4T1 tumors. This study measured the effect of repeat administration of nontargeted lipoplexes on clearance, cytokine/chemokine response, plasmid distribution, reporter gene expression, and liver toxicity. We do not observe a refractory period or a statistically significant difference in blood clearance between the first administration and subsequent injections of this lipoplex formulation, consistent with the absence of a cytokine/chemokine response. However, we do see a significant effect on both plasmid accumulation and expression, an enhancement of 26-fold and 10-fold in tumor plasmid levels and expression, respectively, after 4 injections as compared to that after a single injection. In addition, *in vivo* imaging suggests that expression in other organs had diminished rapidly 72 h after each administration, in contrast to relatively constant expression in the tumor. Taken together, the findings indicate that gene delivery to tumors can be dramatically enhanced by employing repetitive administration.

Introduction

Hereditary diseases were thought to be incurable prior to the demonstration that DNA could be successfully delivered to cells. While the use of calcium phosphate for transfection was utilized for early laboratory experiments, the demonstration that cationic lipids were capable of delivering DNA much more efficiently brought hopes that similar approaches might one day allow genetic diseases to be corrected.[1-3] This hope has recently been bolstered by the demonstration of specific gene splicing by the CRISPR system.[4] It is now clear that RNA (micro ribonucleic acid, messenger ribonucleic acid, siRNA) can also be used to alter gene expression, but effective treatment still depends on developing delivery approaches that are sufficiently targeted and efficient such that expression and silencing can be adequately regulated to impart a therapeutic effect. Although many different nucleic acid-based treatments have been tested in clinical trials, very few have been approved for use as pharmaceutical products. However, new approaches to nucleic acid-based therapy continue to evolve and are currently being tested in clinical trials.

With the exception of Glybera®, which is purported to be curative after a single treatment, it is thought that current nucleic acid-based medicines will need to be administered repetitively in order to achieve and maintain therapeutic effects. Prior to clinical trials, extensive animal testing is performed, and dosing studies typically employ repetitive administration to simulate dosing that would likely be employed in the clinic.[5-7] However, initial animal studies designed to assess pharmacokinetic parameters, delivery efficiency, and toxicity typically employ a single administration to evaluate the merits of a particular approach or strategy before continuing with further animal testing.[8-11]

Decades of work with soluble small molecule therapeutics has led to our conventional understanding of pharmacokinetic profiles, that is, adsorption, distribution, metabolism, and excretion. According to this classic paradigm, bioavailability is correlated with drug levels in the blood, and the therapeutic effect is abolished after clearance from the blood. In this scenario, repeat administration is utilized to maintain blood levels of the drug within the range that corresponds with the therapeutic effect, that is, the "therapeutic window."[5] However, it is clear that gene-based therapies will need to be taken up into cells prior to having a therapeutic effect, and therefore a significant hysteresis between blood levels and gene expression is expected. The situation is further complicated by the fact that delivery vehicles administered intravenously will

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initially encounter the vasculature endothelium, then be translocated to (and subsequently taken up by) the target cell, and exogenous genes must ultimately access the nucleus after uptake.[12-16] Considering the time needed after transcription for mRNA to be transported into the cytoplasm as well as the residence time of mRNA and protein within the cell, the exogenous gene may be degraded and silenced while the encoded protein responsible for the therapeutic effect continues to be active. It follows that blood levels at any point in time may not accurately reflect the biological activity of nucleic acid-based therapeutics.

Similarly, because the effects of most nucleic acid-based therapeutics will not depend on maintaining high blood levels, repetitive administration is used to progressively enhance deposition in the target tissue with the hope that sufficient levels of nucleic acid accumulate to elicit a therapeutic effect within the target cell.[6,17,18] Indeed, previous studies have utilized repetitive administration of lipoplexes to extend transgene expression[17,18] and achieve enhanced levels of siRNA-induced silencing.[12,14] Under this scenario, it may be expected that each administration results in a constant amount of deposition in tissues. This deposition process is presumably arrested after the nucleic acid is cleared from the blood. As described above, it may be expected that the therapeutic effect resulting from gene expression and silencing may be appreciably delayed as compared to blood clearance; however, it is typically presumed that a single administration should result in a finite level of deposition and distribution in tissues. Assuming that blood clearance is complete before subsequent administration of a repeat dose, it follows that successive dosing would be additive. However, one must account for degradation of the therapeutic nucleic acid that occurs in the tissues prior to deposition of the subsequent dose. Accordingly, 2 doses of a gene delivery system should result in “a maximum” of twice the deposition and expression of a single dose, 3 doses result in a maximum of 3 times that of a single dose, etc. In this way, the effects of the therapeutic nucleic acid should be progressively enhanced by repetitive administrations.

It is well recognized that nonviral gene delivery systems suffer from inefficient delivery as compared to their viral counterparts. In particular, synthetic delivery systems have the potential for repeat administration due to the lack of a specific immune response to the vector, and this strategy allows delivery from nonviral systems to be greatly enhanced.[6,12,14] However, gene delivery studies with nonviral systems have demonstrated that repeat transfection requires a “refractory period” (e.g., 2 weeks) in order to obtain expression from a subsequent dose, and typically the goal of repetitive dosing is simply to maintain expression levels, not increase them.[18] Furthermore, it is generally recognized that both the nucleic acid component as well as the nonviral delivery system can be immunostimulatory, and this can affect delivery after successive administrations.[18] Therefore, we have taken great lengths to diminish the adverse response to our delivery system by utilizing minimal amounts of naturally occurring lipids, reducing the CG dinucleotide instance (CpG) content of the plasmid, and avoiding the use of PEGylation.[19-23] The effects of these particle alterations on the cytokine/chemokine response after repetitive injection was determined, and we also characterize the effects of repeat administration of a lipoplex formulation on clearance, organ accumulation, and liver toxicity. We observe effects on each of these parameters that are not additive and are thereby inconsistent with a conventional pharmacokinetic profile. We feel that these results demonstrate that the correlation among clearance, tissue accumulation, reporter gene expression, and liver toxicity is not straightforward, and is worthy of further investigation. In addition, our *in vivo* imaging demonstrates that reporter gene expression is widely distributed throughout the mouse 24 h after each tail vein injection, but predominantly confined to the tumor at later times (72 h).

Materials and Methods

Lipoplex Preparation and Luciferase Expression

Sphingosine, cholesterol, and 1,2-diarachidoyl-sn-glycero-3-phosphocholine were purchased from Avanti Polar Lipids (Alabaster, AL) and used to prepare liposomes at a 3:2:5 mole ratio (respectively) as previously described.[20] Liposomes were then mixed with a modified (cytomegalovirus removed, ROSA26 added, based on Watcharanurak et al.[24]) pSelect-LucSh (InvivoGen, San Diego, CA) plasmid encoding luciferase at a charge ratio of 0.5.[19,20] These modifications to the plasmid have been shown to prolong expression for weeks to months.[24] The resulting lipoplexes have a diameter of 280.9 ± 10.8 nm, a zeta potential of -24.4 ± 2.9 mV, and were diluted 1:1 (vol/vol) with 12% hydroxyl ethyl starch (MW 250,000; Fresenius, Linz, Austria) prior to administration.[19] The use of hydroxyl ethyl starch at a final concentration of 6% (wt/vol) serves to adjust the tonicity and results in more consistent, but not increased, delivery (unpublished observations). Fifty micrograms of DNA complexed with 0.25 μmole lipid was injected via tail vein as previously described.[19] Each mouse received a series of 4 injections at 3-day intervals. Prior to treatment with lipoplexes, female immunocompetent BALB/c mice 6-10 weeks old were acquired from the Jackson Laboratory (Bar Harbor, ME) and inoculated in each shoulder with one million 4T1 murine mammary carcinoma cells (ATCC #CRL-2539). Luciferase expression was monitored in extracted tissues with Promega Luciferase Assay Reagents (Madison, WI) as previously described.[23] All animal procedures were approved by the University of Colorado Institute for Animal Care and Use Committee in accordance with guidelines from the National Institutes of Health.

Cytokine/Chemokine Response

A separate set of tumor-bearing mice was used to quantify levels of specific cytokines and chemokines after repetitive injection of lipoplexes. In addition to the lipoplex formulation described above, mice were treated with phosphate buffered saline (PBS; negative control), lipoplexes formulated with 5% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine−PEG2000 (Avanti), CpG-containing plasmid (Valentis, Inc.), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP; Avanti) instead of sphingosine, or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as previously described.[19,20,22,25] In these studies, a single mouse was treated with each formulation as described above, and blood was collected in Eppendorf tubes 2 h after the second injection of lipoplexes. Serum samples were obtained per manufacturer's instructions: samples were allowed to clot for 30 min, spun at $2000 \times g$ for 15 min, and serum was recovered (R&D Systems, Minneapolis MN). Samples were assayed for cytokine/chemokine activity via manufacturer's instructions using the mouse cytokine array panel A (#ARY006; R&D Systems). The 2 h time point after the second injection was chosen because that is when cytokine/chemokine response was shown to be maximized in previous studies.[26]

Determination of Plasmid Levels in Tissues

To determine delivery of plasmid DNA to mouse tissues, animals were sacrificed 24 h after the first and fourth intravenous administration of lipoplexes, and their organs were harvested and flash frozen in liquid nitrogen. Organs were then thawed, weighed, and DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit