Published in final edited form as:

Mol Pharm. 2011 October 3; 8(5): 1980–1984. doi:10.1021/mp2002413.

A Single Intrathecal Injection of DNA and an Asymmetric Cationic Lipid as Lipoplexes Ameliorates Experimental Autoimmune Encephalomyelitis

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Abstract

Intrathecal delivery of gene therapeutics is a route of administration that overcomes several of the limitations that plague current immunosuppressive treatments for autoimmune diseases of the central nervous system (CNS). Here we report intrathecal delivery of small amounts (3 µg) of plasmid DNA that codes for an immunomodulatory fusion protein, OX40-TRAIL, comprised of OX40, a tumor necrosis factor receptor, and tumor necrosis factor related apoptosis inducing ligand (TRAIL). This DNA was delivered in a formulated nucleic acid-lipid complex (lipoplex) with an asymmetric two-chain cationic lipid myristoyl (14:0) and lauroyl (12:1) rosenthal inhibitor-substituted compound (MLRI) formed from the tetraalkylammonium glycerol-based compound *N*-(1-(2,3-dioleoyloxy)-propyl)-*N*-1-(2-hydroxy)ethyl)-*N,N*-dimethyl ammonium iodide. Delivery and expression in the CNS of OX40-TRAIL in the mouse prior to onset of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, decreased the severity of clinical disease. We believe this preclinical demonstration of rapid, widespread, and biologically therapeutic nonviral gene delivery to the CNS is important in further development of clinical lipid-based therapeutics for CNS disorders.

Keywords

CNS gene delivery; asymmetric lipids; animal model; experimental autoimmune encephalomyelitis

Introduction

Autoimmune disorders are a unique class of diseases whose clinical complexity and unknown causes make them difficult to treat. Current treatments rely on intensive steroid regimens that produce unfavorable generalized immunosuppression. One therapeutic strategy for experimental autoimmune demyelinating disease of the central nervous system

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(CNS) is systemic immunogene therapy in which the therapy is delivered during disease induction.¹ However, gene therapy suffers from two major problems. Many commonly used gene delivery vehicles, such as viruses, are inherently immunogenic, leading to adverse reactions and limited effectiveness. While nonviral gene therapy systems, such as biolistics, electroporation, or naked DNA administration by hydrodynamics, have reduced immunogenicity, these systems suffer from relatively low transfection efficiency, especially of postmitotic neurons or glial cells.^{2, 3} To attain high efficiency, large amounts of DNA were required for delivery into the CNS, joint space, or other sites of inflammation to provoke a therapeutic response. For example, intracerebral injection of 100 µg of plasmid DNA encoding transforming growth factor-β, interleukin-4, soluble dimeric human tumor necrosis factor (TNF) receptor p75, or interferon-α mixed with lipofectin was needed for a therapeutic effect in an EAE animal model.⁴ Peripheral routes of administration of lipoplexes, such as intramuscular injection, result in uptake of the lipoplexes in different tissues. In a mouse model of EAE, intramuscular inoculation of 50 µg of plasmid DNA (interferon-γ-inducible protein 10, a truncated diphtheria toxin, DT390) was required to penetrate the CNS and delay and alleviate disease.⁵

Delivery of nucleic acids (DNA, mRNA, small RNAs) is most commonly achieved by using formulated complexes with lipids. We used MLRI (Figure 1A),^{6,7,8} an asymmetric C(14)–C(12) cationic lipid variant of the highly active C(14)–C(14) cationic lipid dimyristooxypyl dimethyl hydroxyethyl ammonium bromide,⁹ for the formation of lipoplexes because of the increased efficiency of transfection and lower toxicity of MLRI compared to that observed with symmetric lipids in mice and rats.^{6, 7, 10, 11} In addition, MLRI lipoplexes have been used in nonhuman primates, and the multiplex cytokine data from these experiments showed little immune response in serum or CSF to the lipoplex (Hecker J. et al, unpublished results).

EAE is an animal model for multiple sclerosis caused by an autoimmune response involving pathogenic T cells directed towards the CNS.^{12, 13} OX40, a member of the TNF receptor super family, is found predominantly on activated encephalitogenic T cells in subjects with EAE.^{14, 15} OX40 ligand (OX40L)–/– mice are resistant to the induction of EAE.¹⁶ Furthermore, treatment with a monoclonal antibody to OX40L ameliorates disease.¹³ A fusion protein comprised of fibroblast growth factor-inducible 14 (Fn14), a receptor for blocking pro-inflammatory signaling, and TNF-related apoptosis-inducing ligand (TRAIL), a ligand capable of inhibiting pathogenic T cells, ameliorated EAE in mice.¹⁷ Based on the results from Fn14-TRAIL, another chimeric immunomodulatory protein, OX40-TRAIL, was constructed. Preliminary data indicate that OX40-TRAIL has immunomodulatory effects in a delayed-type hypersensitivity immunologic animal model (Yellayi et al., unpublished results). In the present study, mice receiving an intrathecal injection of plasmid, pOX40-TRAIL/ND (DNA encoding OX40-TRAIL cloned into the pND vector), as a lipoplex with MLRI during disease induction had decreased severity of EAE and reduced inflammatory cell infiltrates beneath the meninges compared to mice receiving pND lipoplex. Such therapeutic effects on EAE were achieved by injecting small quantities (3 µg/subject) of lipid-DNA complexes at much lower doses than previously effective doses.

Experimental Section

Luciferase DNA Vector

Construction of the expression vector for this study containing the CDNA sequence for firefly luciferase (pNDluc) from the firefly *Photinus pyralis* (Promega, Madison, WI) was described in previous studies.^{6, 8, 11} pNDluc MLRI lipoplex was administered intrathecally in five C57BL/6 mice, and brain sections were retrieved 5 days postinjection as described below.

Formulation of Lipid:DNA Complexes

Lipid-DNA complexes were evaluated previously by extensive in vitro and in vivo transfection. To obtain the maximal luciferase expression in these transfections, the charge ratio, dose, formulation time, concentration, incubation time and temperature, and lipid composition were optimized first in vitro and then in vivo.

Preparation of MLRI was described in previous studies.^{6, 8, 11} Briefly, chloroform was added to dry MLRI and mixed with dioleoylphosphatidyl-ethanolamine (DOPE) 50:50 in chloroform. The solution was vortexed, aliquoted into glass vials, dried, and stored at 0°C. After rehydrating the MLRI:DOPE film at 4°C, cDNA was added to the lipid preparation and incubated for 30 min at 37°C before intrathecal delivery. Stability testing revealed that the transfection ability of the rehydrated MLRI:DOPE preparation improved for several months. The lipid-DNA complexes were most active if used within 1–2 h after the start of incubation.

Induction of EAE and intrathecal gene administration

Eight-week old female C57BL/6 (n = 17) mice were immunized subcutaneously with 300 µg myelin oligodendrocyte glycoprotein (MOG) peptide fragment (amino acids 38–50) in 200 µL of phosphate-buffered saline (PBS) and incomplete Freund's adjuvant 1:1 containing a final concentration of 2.5 mg/ml of *Mycobacterium tuberculosis* H37RA strain. The subcutaneous injection was divided over two injections of 100 µL each, one on either flank. Pertussis toxin (100 ng in 200 µL of PBS) was administered intraperitoneally immediately and again 48 h later to facilitate induction of disease. On Day 8 after MOG challenge, one group of mice (n = 9) received a single intrathecal injection of lipoplexes containing the plasmid vector pND, and a second group (n = 8) received lipoplexes containing pOX40-TRAIL/ND. Each type of lipoplex consisted of 3 µg of DNA in 1 µL PBS mixed with 9 µL of MLRI to achieve a final concentration of 333 ng/µL of DNA-MLRI lipoplex. This 10-µL mixture was slowly injected into the cisterna magna using a Hamilton syringe.

Mice were monitored daily for 30 days postinjection and assigned a clinical score based on the following scheme: 0, no clinical signs; 1, tail weakness; 2, weak hind limbs; 3, paralyzed hind limbs; 4, weak forelimbs and paralyzed hind limbs; or 5, moribund or dead, as previously described.¹⁸ To prevent death from dehydration, mice with a score of 3 or greater received Transgel™ (Charles River, Wilmington, MA) with chow on the floor of each mouse cage. Mice without motor deficits (i.e., clinical scores of 1–2) that were able to get to food and water did not receive Transgel™. Clinical scores for each mouse in the two experimental groups were added together and then averaged to yield mean cumulative clinical scores. Statistical analysis of the clinical scores was done with the nonparametric test, Mann-Whitney test. All mice were euthanized at study end (day 30) when the clinical criteria of euthanasia (a clinical score of 4 for 3 consecutive days) was not reached.

Immunohistochemical detection of OX40 and luciferase

Spinal cord tissues were drop-fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned to a 4-µm thickness. Paraffin was removed from tissues sections on glass slides with xylene, and the sections were rehydrated in a series of alcohol washes. Heat-induced epitope retrieval was performed using citrate (pH 6.0) at 100°C for 25 min. Immunohistochemical detection of OX40 was done as described previously.¹⁹ Following intrathecal administration of pNDluc lipoplexes in five mice, immunohistochemical detection of luciferase from brain sections on day 5 postinjection was done as described previously using a polyclonal antibody against firefly luciferase (Promega, Madison, WI).²⁰

Results and Discussion

By previous sequential bioluminescent imaging, we have shown widespread distribution of transgene luciferase in the CNS for 5 or more days following intrathecal/intracranial injection of pNDluciferase DNA-MLRI lipoplex into the cisterna magna of mice¹⁰ and rats.⁸ In this study, immunohistochemical staining of brain sections on day 5 indicated luciferase expression in the cells of the ependyma as well as the subventricular zone in the mesencephalon (Figure 1C). In addition, we also investigated the expression of OX40 by immunohistochemistry in lesions in mice with active EAE. OX40 expression was selectively increased in inflammatory cells (lymphocytes, macrophages), glial cells, and endothelial cells at inflammatory sites in the spinal cord (Figure 3C). In healthy unchallenged mice, OX40 was not expressed (Figure 3D).

Administration of plasmid, pOX40-TRAIL/ND, as a lipoplex with MLRI into the mouse cisterna magna on day 8 after induction of EAE but prior to the onset of disease decreased the subsequent severity of the disease (Figures 2A and 2B). Mean cumulative score was significantly decreased ($P \leq 0.05$) in pOX40-TRAIL/ND lipoplex-treated mice compared to that observed in pND lipoplex-treated mice at day 17 postinjection. In spinal cord tissue, inflammatory cells were appreciably reduced beneath the meninges following pOX40-TRAIL/ND lipoplex administration (Figure 3B) compared to that observed following administration of lipoplexes containing the pND vector only (Figure 3A).

Injection of DNA-cationic lipid lipoplexes directly into cerebrospinal fluid facilitates fusion with the plasma membrane and uptake of DNA by astrocytes and oligodendrocytes.^{21, 22} Such local delivery of lipoplexes was effective in a rodent model of Parkinson's disease.²² Injection of small quantities (10 μ L of 333 ng/ μ L) of DNA-MLRI lipoplexes intrathecally resulted in widespread expression of the reporter protein luciferase for at least 5 days. Using the same DNA-MLRI lipoplex in healthy rat brains, a similar (7 days) duration of luciferase activity was previously observed.⁸

In addition, pretreatment with OX40-TRAIL DNA lipoplex prior to the onset of EAE decreased the severity of subsequent disease and infiltration of lymphocytes and macrophages to inflammatory sites. Similar to the fusion protein FN14-TRAIL, OX40-TRAIL probably acts to block OX40L actions and activate TRAIL receptor intracellular signaling pathways. Costimulatory signals by OX40L on antigen-presenting cells promote CD4⁺ cell clonal expansion, and the resulting cytokine responses attract activated T cells and inflammatory cells to sites of inflammation²³ and exacerbate EAE.^{13, 15, 16, 24} Blockade of OX40L actions by the OX40-TRAIL fusion protein presumably converts proliferative OX40L signals to inhibitory TRAIL signals. TRAIL ameliorates EAE in part by down regulating the expansion of CD4⁺ cells (e.g., apoptosis, functional inactivation, inhibition of growth) and inhibiting cytokine responses.^{17, 25} In addition, TRAIL suppresses autoimmunity through the proliferation of regulatory T cells.^{17, 25, 26}

Although the therapeutic effect, as measured by mean cumulative clinical scores, was significant for only a relatively short duration (7–10 days following disease onset), MLRI lipoplex appreciably reduced the amount of DNA needed to achieve therapeutic endpoints. Future studies will be directed toward prolonging the expression of the transgene OX40-TRAIL within the CNS and enhancing the efficiency of transfection of postmitotic neurons. The feasibility of prolonged expression of the pOX40-TRAIL/ND lipoplex depends on low toxicity. Although targeted intrathecal administration of lipoplexes bypasses some of the toxicity associated with systemic administration,²⁷ future studies should be performed evaluating the toxicity of the pOX40-TRAIL/ND lipoplex, including immune or inflammatory responses.

In summary, we showed for the first time that widespread expression of the reporter protein luciferase for at least 5 days is possible following intrathecal injection of small quantities of pNDluc-MLRI lipoplexes. Such distribution of an expressed protein is critical for future clinical applications using vectors that deliver therapeutic genes. In addition, first time delivery of lipoplexes with transgenes for the novel recombinant fusion protein OX40-TRAIL prior to the onset of EAE significantly decreased the mean cumulative clinical scores and infiltration of cells to inflammatory sites. Further characterization of the effects of the fusion protein OX40-TRAIL on activated T cells in diseases that involve expansion of such cells may provide an increased understanding of the mechanism and utility of this fusion protein.

Acknowledgments

We thank Dr. Mark Tykocinski for providing the fusion protein (OX40-TRAIL) plasmid. We also thank Shaomin Zou and Yvette Liu for their technical assistance. This study was supported by NIH NINDS R01 NS046591 (JGH).

Abbreviations used

CNS	central nervous system
DNA	deoxyribonucleic acid
DOPE	dioleoylphosphatidyl-ethanolamine
EAE	experimental autoimmune encephalomyelitis
Fn14	fibroblast growth factor-inducible 14 receptor
MLRI	myristoyl (14:0) and lauroyl (12:1) rosenthal inhibitor-substituted compound formed from the tetraalkylammonium glycerol-based compound <i>N</i> -(1-(2,3-dioleoyloxy)-propyl)- <i>N</i> -1-(2-hydroxy)ethyl)- <i>N,N</i> -dimethyl ammonium iodide
MOG	myelin oligodendrocyte glycoprotein
OX40L	OX40 ligand
PBS	phosphate-buffered saline
RNA	ribonucleic acid
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand

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Fig. 1A **Fig. 1B**

Figure 1.

Figure 1A. Structure of MLRI.

Figure 1B. Schematic of an animal model for therapeutic CNS gene delivery directly into the cerebrospinal fluid. Insert: Schematic of lipid bilayer. The actual lipoplex is a more heterogeneous structure, with condensed nucleic acid interspersed with hydrocarbon domains.

Figure 1C. Immunohistochemical demonstration of luciferase expression within the subependymal region (arrow) of the third ventricle 5 days post intrathecal injection of DNA encoding for luciferase in combination with MLRI. Brains were fixed in neutral buffered formalin, and immunohistochemistry was performed as described in materials and methods.

Fig. 2A

Fig. 2B

Figure 2.

Figure 2A. Suppression of EAE by intrathecal expression of OX40-TRAIL. One group of MOG₃₈₋₅₀-challenged mice (n = 8) was treated with a single intrathecal injection of pOX40-TRAIL/ND lipid- complexes on day 8 postchallenge. A second group of challenged mice (n = 9) were treated with pND lipid complexes. Mice were assigned clinical scores daily. The y-axis shows the mean clinical scores for both groups of mice.

Figure 2B. Daily clinical scores were added together for each individual mouse an experimental group described in Figure 2A and then averaged to yield mean cumulative clinical scores. * significant difference between the group receiving pND empty vector lipoplexes and the group receiving pOX40-TRAIL/ND lipoplexes ($P \leq 0.05$). Statistical analysis of the clinical scores was done by Mann-Whitney nonparametric test.

Figure 3. Histopathological section of spinal cords stained with hematoxylin and eosin

A. Following administration of empty plasmid vector pND lipoplexes and MOG, inflammatory cells composed of lymphocytes, macrophages, and occasional neutrophils infiltrated the white matter tracts, resulting in diffuse nonsuppurative myelitis.

B. Mice treated with OX40-TRAIL-MLRI and MOG had appreciably reduced number of the inflammatory cells predominantly present beneath the meninges (subpial).

C. Following MOG administration, OX40 protein was expressed by mononuclear cells, endothelial cells, and glial cells in an inflammatory site of the spinal cord.

D. Non inflamed spinal cord from an unchallenged control mouse expressed no OX40 protein.