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Combined Local Pulmonary and Systemic Delivery of *AT2R* Gene by Modified TAT Peptide Nanoparticles Attenuates Both Murine and Human Lung Carcinoma Xenografts in Mice

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ABSTRACT

To evaluate the potential of cell-penetrating peptide-based delivery of apoptosis-inducer gene in cancer therapy, a modified HIV-1 TAT peptide (dimerized TAT peptide, dTAT) was studied. The dTAT and plasmid DNA (pDNA) complexes (dTAT-pDNA) were condensed using calcium chloride (dTAT-pDNA-Ca^{2+}). This simple nonviral formulation approach showed high levels of gene expression *in vitro* without any cytotoxicity. In mouse studies, a single intratracheal (IT) aerosol spray or 2 intravenous (IV) injections of the dTAT, apoptosis-inducer gene, angiotensin II type 2 receptor (AT2R), and Ca^{2+} complexes (dTAT-pAT2R-Ca^{2+}) significantly attenuated the acutely growing mouse Lewis lung carcinoma allografts in mouse lungs. Furthermore, single IT ($p = 0.054$) and the combination of IT and IV ($p < 0.05$) administrations of dTAT-pAT2R-Ca^{2+} markedly attenuated slowly growing and relatively large-sized H358 human bronchioloalveolar carcinoma xenografts in mouse lungs. These results indicate that the dTAT-pDNA-Ca^{2+} effectively delivered the gene to cancer cells by either IT or IV administration although the local pulmonary delivery of the dTAT-pAT2R-Ca^{2+} showed more effective growth inhibition of orthotopic lung cancer grafts. Thus, the present study offers preclinical proof of concept that a dTAT-based nonviral gene delivery method *via* IT administration may be an effective lung cancer gene therapy.
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Introduction

Although lung cancer prognosis has improved because of advances in diagnosis and early surveillance, more than half (57%) of the patients are diagnosed at an advanced stage and the 1- and 5-year survival rate is 26% and 4%, respectively.[1] Gene therapy has become a promising approach for the treatment of numerous diseases including cancers that are considered incurable.[2] Although the lungs may be accessed *via* intravenous (IV) administration or inhalation, biologic obstacles continue to slow the advance of lung cancer therapies.[3] The sensitivity to enzymatic degradation and the poor permeability of nucleic acids considerably complicate the development of most gene therapy strategies. Thus, a successful gene therapy strategy largely depends on the design of efficient and safe vectors.[4-8]

A great deal of effort has been devoted to developing successful viral and nonviral gene delivery systems capable of improving upon a variety of limitations, including *in vivo* instability, low gene transfection efficiency, and toxicity.[9] Viral gene therapy has dominated clinical applications, but nonviral gene therapy has been given significant attention as a gene therapy method because of the low cost, ease of synthesis, and potential for lower immunogenicity in comparison to viral methods. Plasmid DNA (pDNA) or small interfering RNA (siRNA) combined with cationic lipids (lipoplexes) or polymers (polyplexes) to form complexes is the most commonly employed nonviral gene method.[9-16] Nonviral vectors often suffer from a lower transfection efficiency compared to viral vectors,[2] yet numerous strategies have been put forward to advance nonviral gene delivery.

Cell-penetrating peptides (CPPs) appear to be a particularly promising component of nonviral gene therapies. CPPs consist

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of ≤30 amino acids (cationic or amphiphilic in nature), which can mediate transport across the cell membrane.[17-19] The charge, size, and molecular weight of CPPs have a significant role in condensing and delivering genetic material to the target cells.[17] Two main approaches have been investigated: covalent coupling (chemical linkage) and noncovalent coupling (electrostatic interactions) of CPPs with pDNA or siRNA.[20-23] When using noncovalent coupling, strong binding between CPPs and pDNA is essential to stabilize the resulting complexes and to achieve high levels of gene expression; however, binding between CPPs and pDNA must not be too strong, to facilitate the release of the cargo after cellular uptake.

One particular CPP of interest is TAT derived from HIV (RKKRRQRRR), which has been demonstrated to translocate across cell membranes.[17,20,24,25] Here, a longer "double" TAT (dTAT, RKKRRQRRRHRRKKR) was investigated as a potential carrier for pDNA. When used alone, the transfection efficiency of the dTAT-pDNA complexes is quite low; however, calcium has been shown to regulate the delicate balance of binding strength within polyplexes.[9,17,20] The addition of calcium chloride ($CaCl_2$) to the dTAT-pDNA complexes directly affects particle size and gene expression.[9]

In the present study, a simple formulation (dTAT-pDNA-Ca^{2+} complex) was optimized using 3 different human cell lines: (1) A549 (a lung cancer cell), (2) HeLa (a cervical cancer cell), (3) HEK-293 (a virus-immortalized kidney cell) and one mouse cell line, Lewis lung carcinoma (LLC, a lung cancer cell) using a luciferase-reported pDNA (pLUC) to evaluate transfection efficiency. Next, the formulation was tested *in vivo* (LLC tumor-bearing mice) using apoptosis-inducer gene pDNA: angiotensin II type 2 receptor pDNA (pAT2R). AT2R is recognized to inhibit cell proliferation and stimulate apoptosis in various cells (e.g., neuronal, endothelial, prostate, and lung cancer cells).[3,25,26] The dTAT-pAT2R-Ca^{2+} complexes were administered intravenously or *via* intratracheal (IT) aerosol spray to determine lung cancer attenuation in acutely growing murine lung carcinoma (LLC) and slowly growing human bronchioloalveolar carcinoma (H358) graft-bearing mice.

Materials and Methods

Materials

pDNA encoding firefly luciferase (pGL3, 4818 bp) was obtained from Promega (Madison, WI). pDNA encoding human AT2R (agtr2 pcDNA3.1þ) was obtained from the UMR cDNA Resource Center (University of Missouri, Rolla, MO). The pDNA purity level was determined by UV spectroscopy and agarose gel electrophoresis. dTAT (RKKRRQRRRHRRKKR; molecular weight = 2201.7 Da) peptide was purchased from Biomatik USA, LLC (Wilmington, DE). Branched polyethylenimine (PEI, 25 kDa) was obtained from Sigma-Aldrich (Milwaukee, WI). A549 cell line (human lung carcinoma), LLC (mouse Lewis lung carcinoma), HeLa cell line (human cervix adenocarcinoma), and H358 cell line (human bronchioloalveolar carcinoma) were obtained from American Type Culture Collection (Rockville, MD). HEK-293 (human embryonic kidney) cell line was a gift from Dr. Nikki Cheng (University of Kansas Medical Center, Lawrence, KS). Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) was purchased from Fisher Scientific (Pittsburgh, PA). Mouse serum albumin (MSA) and glucose were obtained from Sigma-Aldrich.

Preparation of the dTAT-pDNA-Ca^{2+} Nanoparticles

For the *in vitro* studies, the dTAT-pDNA nanoparticles were prepared by adding 15 μL of dTAT solution (N/P 10, different polymer nitrogen to pDNA phosphate [N/P] ratios) to 10 μL (0.1 μg/μL) of pDNA (Tris-acetate-EDTA [TAE] buffer [1x] was used as a solution for DNA storage), followed by fast pipetting for 20 s. At that point, 15 μL of identified molarity (e.g., 50, 300, and 600 mM) $CaCl_2$ was added and mixed by fast pipetting. The total volume was 40 μL. After preparing the nanoparticles, they were stored at 4°C for 20-25 min. For the mouse studies with IV administration, the dTAT-pDNA nanoparticles were prepared by adding 60 μL (0.88 μg/μL) of dTAT solution to 40 μL (0.1 μg/μL) of pDNA (pAT2R or pLUC; TAE buffer [1x] was used as a solution for DNA storage), followed by fast pipetting for 20 s. At that point, 60 μL of identified molarity (100 mM) $CaCl_2$ was added and mixed by fast pipetting. Then, 40 μL of MSA (1%) was added to the solution. The total volume was 200 μL. After preparing the nanoparticles, they were stored at 4°C for 20-25 min. For the mouse studies with IT administration, the dTAT-pDNA nanoparticles were prepared by adding 15 μL (0.88 μg/μL) of dTAT solution to 10 μL (0.1 μg/μL) of pDNA (pAT2R or pLUC; TAE buffer [1x] was used as a solution for DNA storage), followed by fast pipetting for 20 s. At that point, 15 μL of identified molarity (100 mM) $CaCl_2$ was added and mixed by fast pipetting. Then, 10 μL of glucose (10%) was added to the solution. The total volume was 50 μL. After preparing the nanoparticles, they were stored at 4°C for 20-25 min.

Preparation of the PEI-pDNA Nanoparticles

The PEI-pDNA nanoparticles were prepared by adding 15 μL of PEI solution (N/P 10) to 10 μL (0.1 μg/μL) of pDNA followed by fast pipetting for 20 s. After preparing the nanoparticles, they were stored at 4°C for 20-25 min. The nanoparticles were prepared immediately before each experiment.

Agarose Gel Electrophoresis

The nanoparticles were prepared as defined previously and subsequently: 4 μL of TAE buffer was added to the nanoparticles. Then, 4 μL of SYBR® Green 1 was mixed with the nanoparticles. Afterward, the mixture was stored at 4°C for 20-25 min. After storage, 7 μL of 6X DNA Loading Dye was added. A 1-kb DNA ladder was used as a reference marker. The mixture of the solutions was loaded onto a 1% agarose gel and electrophoresed for 30 min at 110 V.

Size and Zeta Potential

The particle size (effective diameter [nm]) of the dTAT-pDNA nanoparticle with or without calcium chloride was determined by dynamic light scattering (Brookhaven Instruments, Holtsville, NY). The zeta potentials of the nanoparticles were measured by Zeta PALS dynamic light scattering (Brookhaven Instruments). All samples intended for particle size measurements were prepared using PBS, nuclease-free water (NFW), and serum-Free media (SFM). All samples intended for zeta potential measurements were prepared using KCL (1 mM).

Cell Culture

A549, HeLa, LLC, HEK-293, and H358 cell lines were grown in F-12K Nutrient Mixture media (Kaighn's modified with L-glutamine; Mediatech, Inc., Manassas, VA) for A549; Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Grand Island, NY) for HeLa, LLC, and HEK-293; and RPMI 1640 medium (Mediatech, Inc.) with 1% (v/v) penicillin/streptomycin and 10% (v/v) fetal bovine serum at 37°C in 5% CO_2 humidified air.

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