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Protein impurities from cell culture dramatically impact transduction efficiency of polymer/virus hybrid vectors

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Polyethylenimine (PEI) was used recently with murine leukemia virus-like particles (MLV-VLPs) to produce a hybrid vector that possesses advantages over the native virus; the transduction efficiency of this vector, however, was less than the transduction efficiency of the native virus. The cause of the reduced efficiency was hypothesized to be related to the involvement of proteins in PEI/MLV-VLP complex formation and overall complex size. To test the hypothesis and potentially improve the efficiency of the hybrid vector, ultracentrifugation and size exclusion chromatography were used to purify MLV-VLP and to study the effect of proteins in cell culture medium on complex formation. Based on dynamic light scattering and electron microscopy, complexes formed from the purified MLV-VLPs were smaller, but surprisingly, less efficient than complexes formed from unpurified MLV-VLPs. The addition of protein to purified MLV-VLPs showed that the initial efficiency could be restored and that the purification strategy was not inactivating the MLV-VLPs. Further, by optimizing the amount of protein added to the purified MLV-VLPs, the level of transduction by PEI/MLV-VLP improved 1.6-fold. Particle characterization showed a correlation between the size of the PEI/MLV-VLP complex and the transduction efficiency, which is likely a result of greater sedimentation and cell contact during in vitro studies.

1. Introduction

Design of safe and efficient gene delivery vectors has been a major focus of gene therapy research and over the past two decades, viral and synthetic vectors have both played significant roles in the delivery of therapeutic genes (Blaese et al., 1995; Hyde et al., 2000; Rosenberg et al., 1990; Wiley, 2011). Despite having high efficiency and long-term transgene expression, many viral vectors are associated with adverse immune responses (Chen et al., 2003; Nayak and Herzog, 2010), oncogenicity (Montini et al., 2009), issues with tropism, sensitivity to freeze–thaw cycles (Higashikawa and Chang, 2001) and ultracentrifugation (Andreadis et al., 1999), excessive costs related to production and purification, and limited capacity to transport therapeutic genes (Lai et al., 2010). Synthetic vectors, such as cationic liposomes and polymers, are less immunogenic, less pathogenic and can be targeted to particular cells by using targeting ligands (Leamon et al., 1999; Reddy et al., 2002; Sunshine et al., 2009); however, synthetic vectors currently lack the necessary efficiency required of a clinically-relevant gene delivery vector. The poor efficiency of synthetic vectors is often attributed to the lack of efficient endolysosomal escape (Meyer et al., 2008; Nishiyama et al., 2006; Zhou et al., 2007), poor biodegradability (Luten et al., 2008), formation of large aggregates in the presence of serum, and cytotoxicity (Moghimi et al., 2005). The objective is to overcome these current limitations by designing hybrid vectors that will combine the desired characteristics of both viral and synthetic vectors.

The idea of combining the features of a polymer or lipid with a virus has emerged recently as a viable gene therapy strategy. For example, previous studies demonstrated that modification of adenovirus with polyethylene glycol (PEG) could impart traits associated with PEG and reduce the susceptibility of the virus to both adaptive and innate immune inactivation (Croyle et al., 2001; Mok et al., 2005; O'Riordan et al., 1999). Similarly, studies have shown that the ability of cationic lipids and polymers to transport cargo into cells could be used to restore the infectivity of non-infectious virus-like particles (VLPs). Sharma et al. (1997) showed that non-infectious VLPs, based on the murine leukemia virus (MLV), could be made infectious by the addition of lipofection reagents, and Ramsey et al. (2010) formed infectious polymer/MLV-VLP complexes using the same non-infectious MLV-VLPs and either poly-L-lysine (PLL) or polyethylenimine (PEI).

While these early studies demonstrated the potential benefits of a hybrid vector approach, the particles produced by Ramsey et al. demonstrated low transduction efficiency (~5%) compared to the native virus and resulted in relatively large (~1 μm) particles. The particle morphology, as determined from transmission electron

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micrographs, appeared to be large polymer/MLV-VLP aggregates which were thought to be a result from the negatively-charged serum proteins in the MLV-VLP supernatant interacting with the cationic polymer.

The purpose of the current study was to evaluate the effect of proteins in cell culture medium on the formation of polymer/MLV-VLP complexes and their transduction efficiency. The initial hypothesis was that smaller and more efficient hybrid vector particles could be formed from MLV-VLPs that had been purified from cell supernatant, which contains serum proteins and excreted cellular proteins. Smaller hybrid vector particles were expected to enhance internalization and gene delivery efficiency.

2. Materials and methods

2.1. Cell lines and plasmids

The packaging cell line GP-293Luc was used for production of virus and MLV-VLPs, and human embryonic kidney cells (HEK-293) were used to evaluate transduction efficiency. HEK-293 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA), and the GP-293Luc cell line was purchased from Clontech Laboratories Inc. (Mountain View, CA). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) from Sigma–Aldrich (St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and cultured at 37 °C in 5% CO_2.

The plasmid pCMV-VSV-G (Addgene plasmid 8454), which encodes for the vesicular stomatitis virus G envelope glycoprotein (Stewart et al., 2003) was generously provided by Dr. Robert A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA) through the non-profit plasmid repository Addgene.

2.2. Polymers

Branched PEI (molecular weight (MW): 750-kDa) and chondroitin sulfate C (CSC) (shark cartilage, MW: 63-kDa) were purchased from Sigma–Aldrich (St. Louis, MO). A stock solution of PEI was prepared in ultrapure water at a concentration of 10 mg/ml and stored at 4 °C. Stock solutions of CSC were prepared in ultrapure water at concentrations of 1 mg/ml and 10 mg/ml and stored at 4 °C.

2.3. Production of viruses and virus-like particles

Retrovirus pseudotyped with vesicular stomatitis virus G envelope glycoprotein (VSV-G) was produced by transfecting GP-293Luc cells with the pCMV-VSV-G plasmid. The cells were seeded in a 10 cm dishes at a seeding density of 1×10^5 cells/cm^2 18-24 h prior to transfection so as to achieve ~90% confluency at the time of transfection. The cells were transfected with 24 μg of the plasmid DNA using 60 μg of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) following the standard protocol. Briefly, 24 μg of VSV-G plasmid were diluted in 1.5 ml of DMEM without serum and mixed gently. Similarly, 60 μg of Lipofectamine™ 2000 were diluted in 1.5 ml of DMEM without serum. After a 5 min incubation period, the Lipofectamine™ 2000 solution was added dropwise to the plasmid DNA solution while gently mixing. The mixture was then incubated for 20 min at room temperature to form lipoplexes. To prepare for the addition of the lipoplexes, the cell medium in the 10 cm dishes was changed to serum-free medium. After the 20 min incubation period, the 3 ml solution of lipoplexes was added to the 10 cm dish containing serum-free medium. The transfection medium was replaced with the complete growth medium 4 h after transfection. Viruses were collected after 48 h and filtered through a 0.45 μm polyethersulfone syringe filter from Whatman (Florham Park, NJ). Filtered viruses were used immediately.

Envelope-free MLV-VLPs were produced from the GP-293Luc cell line, which was seeded at a cell seeding density of ~2.0×10^4 cells/cm^2 on a 10 cm dish. The cells were cultured for 3 days at 37 °C and 5% CO_2. After 3 days, the supernatant was collected and filtered through a 0.45 μm polyethersulfone syringe filter. Filtered MLV-VLPs were used immediately.

2.4. Concentration of virus

After filtration, virus or MLV-VLP supernatant from five 10 cm dishes was pooled and equally distributed between two ultracentrifuge tubes. Using an SW28 rotor, ultracentrifugation was performed at 100,000 × *g* (24,000 rpm) and 4 °C for 2 h on a Beckman L8-70M ultracentrifuge. The supernatant was aspirated and the virus pellet was resuspended in 5 ml of serum-free DMEM to produce a concentration 10-fold greater than the starting concentration.

2.5. Nuclease digestion

DNase digestion was performed with Benzonase® nuclease which was obtained from Sartorius Stedim (Bohemia, NY). An amount of 200 units of Benzonase® was added to 1 ml of concentrated virus or MLV-VLP supernatant and incubated at room temperature for 1 h. The sample was then loaded onto a size exclusion chromatography (SEC) column to separate the virus particles from the proteins and digested nucleic acids.

2.6. Size exclusion chromatography

Protein A Sepharose CL-4B, from Sigma–Aldrich (St. Louis, MO), was packed in a glass chromatography column (XK16/40). After 3 ml of the nuclease-digested viral or MLV-VLP supernatant was loaded onto the column, TEN buffer was used to elute the various fractions. Fractions of 1 ml were collected from the column, and the absorbance of each fraction was measured at a wavelength of 280 nm using a Cary UV–Visible Spectrophotometer. The purification procedure was performed at 4 °C. After each round of purification, the column is flushed with 50 ml of 10% ethanol and 50 ml of 1 N NaOH and then equilibrated with 150 ml of TEN buffer at a pH of 7.4.

2.7. Hybrid vector formation and transduction

Complex formation and all transduction studies were performed under similar conditions. Based on the fractional recovery of MLV-VLPs and the sample volume, the concentration of MLV-VLP (MLV-VLPs/μl) was normalized to the initial sample concentration. The PEI stock solution was added dropwise to MLV-VLP supernatant while gently vortexing to form PEI/MLV-VLP complexes. A ratio of 10 μg of PEI to 100 μl MLV-VLP supernatant was used to form the complex except in the case of the ratio optimization studies. After addition of the polymer, the solution was incubated for 2 h at room temperature before being used to infect HEK-293 cells, which were seeded in 6-well plates 18–24 h before transduction at a seeding density of 1×10^5 cells/cm^2 so as to be 70–75% confluent at the time of transduction. Before infecting the HEK-293 cells with PEI/MLV-VLP complexes, the growth medium was replaced with serum-free DMEM. The virus or complexes were then added to each well, and the plates were stored at 37 °C. The cells were exposed to the virus or PEI/MLV-VLP complexes for 4 h, after which the serum-free transduction medium was replaced with complete growth medium.