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GENE DELIVERY

A top-down approach for construction of hybrid polymer-virus gene delivery vectors

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ABSTRACT

Safe and efficient delivery of therapeutic nucleic acids remains the primary hurdle for human gene therapy. While many researchers have attempted to re-engineer viruses to be suited for gene delivery, others have sought to develop non-viral alternatives. We have developed a complementary approach in which viral and synthetic components are combined to form hybrid nanoparticulate vectors. In particular, we complexed non-infectious retrovirus-like particles lacking a viral envelope protein, from Moloney murine leukemia virus (M-VLP) or human immunodeficiency virus (H-VLP), with poly-L-lysine (PLL) or polyethylenimine (PEI) over a range of polymer/VLP ratios. At appropriate stoichiometry (75–250 μg polymer/10^6 VLP), the polymers replace the function of the viral envelope protein and interact with the target cell membrane, initiate cellular uptake and facilitate escape from endocytic vesicles. The viral particle, once in the cytosol, efficiently completes its normal infection process including integration of viral genes with the host genome as demonstrated by long-term (at least 5 weeks) transgene expression. In addition, hybrid vectors comprising H-VLP were shown to be capable of infecting non-dividing cells.

1. Introduction

Because of their efficiency, recombinant viral vectors have been employed in nearly 70% of gene therapy clinical trials [1]. Recombinant retroviruses, in particular those based on the murine leukemia virus (MLV), have been studied extensively, especially in *ex vivo* gene transfer protocols [2–4]. On the other hand, viral vectors exhibit severe disadvantages including immunogenicity, pathogenicity and difficulty in cell-specific targeting. Routine clinical implementation of human gene therapy awaits development of new delivery vectors that are both safe and highly efficient [5–7].

A promising strategy for vector design is to re-engineer viruses to exhibit enhanced properties for gene delivery. Both retroviruses and adenoviruses have been genetically engineered to target infection of specific cells. For example, several groups have modified the retroviral envelope protein by fusing or inserting targeting moieties including single-chain antibody fragments or other cell-binding proteins [8]. Similarly, adenovirus displaying cell-specific ligands provides infection of specifically targeted cells [9–11]. Directed molecular evolution has also been employed to enhance viral vectors. Stemmer et al. evolved a murine leukemia virus (MLV) envelope protein for enhanced stability to ultracentrifugation [12] and altered tropism [13], while we recently reported evolution of the MLV protease generating vectors with a 3.5-fold increase in the half-life of infectivity [14]. Schaffer et al. have used directed evolution to produce adeno-associated viral vectors that evade antibody neutralization [15] or exhibit enhanced tropism toward glial cells [16].

An alternative solution employs bottom-up design of non-viral vectors that ideally exhibit the functionality of viruses but are safer and less expensive. Such vectors typically comprise cationic polymers [17] or lipids [18], which are generally considered safe and robust biomaterials. Gene delivery polymers include commercially available materials such as poly-L-lysine (PLL) [19], polyethylenimine (PEI) [20–22] and polyamidoamine dendrimers [23–25], as well as a wide range of materials specifically designed for gene delivery. Regardless of the material(s) chosen, the synthetic vector must efficiently and actively escort the DNA cargo through intracellular barriers including endocytosis, escape into the cytoplasm, transport through the cell and across the nuclear membrane, and release of the DNA [17]. Because synthetic materials inefficiently perform one or more of these functions, non-viral vectors are orders of magnitude less efficient than recombinant viruses and generally lack the efficiency necessary to be clinically relevant.

We propose the construction of hybrid vectors in which recombinant viruses are stripped of essential functional components, and functionality is subsequently replaced using synthetic materials. Thus, we consider this a top-down approach in the sense that the hybrid vectors are built starting from a functional viral vector. Specifically, retrovirus-like particles (VLP) comprise the native viral genome, protein capsid, enzymes and lipid envelope, but lack the viral

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envelope protein. The envelope protein normally serves at least two functions: to recognize and bind the virus to specific cellular receptors, and to mediate entry into the cytosol by fusing the viral lipid bilayer with the cell or endocytic vesicle membrane. In the absence of the envelope protein, VLP lack the ability to attach to the cell and, in the event of non-specific attachment and internalization, VLP lack the ability to escape from endolysosomal compartments into the cytosol. In the hybrid vectors reported here, PLL or PEI is complexed with VLP to provide interaction with the cell membrane and endocytic escape functions. Once in the cytosol, the viral particles are capable of continuing along the infection pathway including efficient cytosolic transport, reverse transcription, nuclear import, and genomic integration (Fig. 1).

We report the formation of these hybrid vectors and investigate the effects of the complex stoichiometry on gene delivery. In addition, the intracellular processing of hybrid vectors was visualized by transmission electron microscopy (TEM). Finally, hybrid vectors were shown to exhibit several potential advantages including stable transgene expression, robustness to common processing conditions, and (when using lentiviral VLP) transduction of non-dividing cells.

2. Materials and methods

2.1. Cell lines and plasmids

Human embryonic kidney cells, HEK-293, were obtained from the American Type Culture Collection (Manassas, VA). The MLV producer cell line, GP-293Luc (Clontech, Mountain View, CA) expresses the MLV viral *gag-pro-pol* genes and a viral packaging sequence encoding neomycin resistance and luciferase reporter genes. Both cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and cultured at 37 °C in 5% CO_2.

The plasmid pMD.M(4070A) codes for the amphotropic MLV 4070A envelope protein and was a gift from R.C. Mulligan (Children's Hospital, Boston, MA). The plasmid pLLRN (Clontech, Mountain View, CA) encodes the retroviral packaging insert which contains the neomycin resistance and luciferase reporter genes. The packaging insert contained in the pLLRN plasmid is the same as that stably expressed by the GP-293Luc packaging cell line. The plasmid pVSV-G encodes for the vesicular stomatitis virus G envelope glycoprotein and was obtained from Clontech. The plasmids pREV2, pCGP, and pH9CMV-bgal were gifts from J.K. Yee (City of Hope, Duarte, CA). The *luc* reporter plasmid was created by removal of the *lacZ* gene from pH9CMV-bgal and insertion of the *luc* gene. The pCGP plasmid produces HIV *gag* structural proteins and HIV *pol* enzymatic proteins. The pREV2 plasmid produces the *rev* accessory protein required for efficient production of HIV viruses, and pH9CMV-luc produces the viral insert with a *luc* reporter gene. Plasmids were grown in DH5α *E. coli* (Gibco BRL, Rockville, MD) and purified using a commercial plasmid purification kit (Bio-Rad, Hercules, CA).

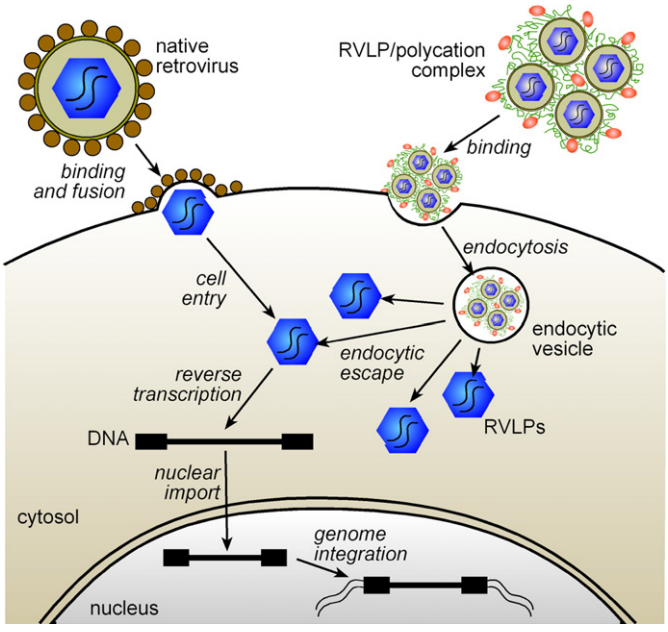

Fig. 1. Schematic comparing the native retrovirus infection process to the proposed pathway of hybrid vector-mediated transfection. The polymer component of the hybrid vector mediates cell binding/internalization and escape from endocytic vesicles. Proviral particles in the cytosol are processed similarly, regardless of whether they were delivered by the native virus or hybrid vector.

2.2. VLP production and hybrid vector transduction

M-VLPs were produced in GP-293Luc cells (~1.5×10^6) seeded in a 10-cm dish. The cells were cultured for three days before the M-VLP-containing supernatant was collected and filtered through a 0.45-μm surfactant-free cellulose acetate syringe filter. The M-VLP concentration was estimated to be 1.0×10^6 i.u./mL. For the production of H-VLP, HEK-293 cells were transfected with 4 μg each of pREV2, pCGP, and pH9CMV-luc. Cells were transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). Transfection medium was replaced after 6 h, and virus supernatant collected after 48 h. H-VLP supernatant was collected every 24 h for four days. VLP were either used immediately or frozen at −80 °C.

Stock solutions of PEI and PLL (Sigma-Aldrich, St. Louis, MO) were prepared in water at 10 mg/ml and stored at 4 °C. Polymer/VLP complexes were formed by dropwise addition of the polymer stock solution to VLP suspension while vortexing. The polymer/VLP mixture was incubated at room temperature for 2 h before being added to the target cells. HEK-293 cells were seeded in 6-well plates 18–24 h before transduction. Immediately before the addition of hybrid vector complexes, the cell growth medium was replaced with 1 mL of serum-free DMEM followed by dropwise addition of 100 μL of the complexes to each well, and the plates were returned to the incubator at 37 °C. The medium containing polymer/VLP hybrid vectors was removed after 4 h and replaced with normal growth medium.

2.3. MLV and VSV-G/HIV production and infection

MLV virions packaging neomycin resistance and luciferase reporter genes were produced from the GP-293Luc packaging cell line seeded in a 10-cm cell culture dish so as to be 80–90% confluent at the time of transfection. The cells were transfected with 24 μg of pMD.M(4070A) using Lipofectamine™ 2000. HIV pseudotyped with VSV-G (VSV-G/HIV) was produced by transfecting HEK-293 cells with 4 μg each of pVSV-G, pREV2, pCGP, and pH9CMV-luc. The virus-containing supernatant was collected after 48 h, filtered through a 0.45-μm surfactant-free cellulose acetate syringe filter and either used immediately or stored at −80 °C.

HEK-293 target cells were seeded in 6-well plates 18–24 h prior to infection. At the time of infection, MLV or VSV-G/HIV supernatant was diluted 10-fold into 1 mL of fresh cell culture medium containing 8 μg/mL Polybrene and used to replace the growth medium on the target cells. After 24 h, the medium was replaced with fresh cell culture medium, and the cells were assayed for luciferase protein after an additional 24 h.

2.4. Polyplex transfection

DNA/polymer complexes were prepared by dropwise addition of 50 μL of 25 kDa PEI (150 μg/mL) or 150–300 kDa PLL (200 μg/mL) in water to 50 μL pLLRN (200 μg/mL). Polyplexes were incubated at 4 °C for 30 min. HEK-293 cells were plated in 6-well plates 18–24 h prior to transfection. The normal growth medium was replaced with serum-free