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Systemic gene transfer of polyethylenimine (PEI)–plasmid DNA complexes to neonatal mice

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ABSTRACT

Non-viral vectors have not been extensively investigated in neonatal mice due to the poor efficiency of the delivery methods available. Understanding the effects of non-viral vectors during early development is vital to develop safe gene therapy treatments where irreversible pathological processes may be avoided by early gene reconstitution. Here we describe a simple and effective method for the systemic administration of non-viral vectors via the superior temporal vein of mouse pups at 1.5 days of age. We show that injection of polyethylenimine (PEI)–complexed plasmid DNA (pDNA) intravenously results in effective transfection in the liver, lung, heart, spleen, brain and kidney. We also investigate the specific targeting of transgene expression to the proliferating neonate liver using a liver-specific plasmid containing a Scaffold Matrix Attachment Region (S/MAR) element, which has previously been shown to confer long-term expression in adult mouse liver. Using bioluminescent imaging, a gradual increase in transgene expression was observed which peaked at days 11–12, before the reduction of expression to background levels by day 25, suggestive of vector copy number loss. We conclude that non-viral vectors can successfully be used for systemic delivery to neonatal mice and that this technique is likely to open a host of early therapeutic possibilities for gene transfer by a range of non-viral vector formulations.

1. Introduction

Gene delivery to juvenile animals could provide a basis for new therapeutic strategies for treatment of various genetic disorders. The early application of gene therapy may also be of medical importance since many genetic diseases require correction earlier in life to avoid irreversible adverse disease manifestation. A further advantage of gene delivery in neonates is the possibility of efficient gene transfer with a lesser quantity of vector due to the smaller body size in early life. In addition, if gene delivery is performed prior to maturation of the immune system, immune reactions against a foreign gene could be pre-empted.

To date gene therapy studies with this aim have been restricted mainly to viral vector systems, which are innately optimized for highly efficient gene transfer. However viral vectors often exhibit unwanted side effects such as random integration and immunogenicity, which compromise their efficacy. The application of lentiviruses to neonatal mice has led to liver tumours [1] and although adeno-associated viruses (AAV) are not associated with acute inflammatory reactions an increased incidence of tumours has also been observed after intravenous injection of AAV2 in neonatal mice [2]. Given that the majority of genetic and metabolic diseases, which are potentially amenable to gene therapy, require therapeutic intervention early in life, the influence of safe non-viral alternatives warrants further investigation. Administration of non-viral pDNA vectors to juvenile mice is still relatively uncommon due to the reduced efficacy of non-viral vectors compared to viruses. Crucially, for most non-viral gene transfer methods *in vivo*, conjugation of pDNA with cationic liposomes and polymers is necessary to condense DNA and form complexes with a high affinity for cell membranes [3,4] as naked DNA is subject to degradation by nucleases resulting in limited gene transfer. Physical methods of gene transfer such as electroporation, microinjection or gene guns are technically difficult in neonatal mice because of problems with access and reduced animal size, which may require specialised equipment. In addition to increased safety, the large cloning capacity of non-viral carriers is highly appealing. Compared to the various routes used when administering viral vectors to target hepatic tissue, non-viral delivery routes have been fairly limited. For example for neonatal liver administrations the only report of pDNA delivery is direct injection of a PEI-conjugated CMV plasmid into hepatic tissue where a 40-fold increase in marker gene expression per mg protein was observed 48 h after gene transfer when compared to adult animals [4], but persistence of expression was not further evaluated. To the best of our knowledge, systemic administration to neonatal mice has not been previously reported using non-viral vectors.

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In this study, we describe a method of delivering non-viral vectors to 1.5-day old mouse pups that involves an intravenous injection of polyethylenimine (PEI)–complexed plasmid DNA (pDNA) (PEI/DNA). PEI/DNA under the control of a ubiquitous promoter at birth results in the successful transfection of cells in the liver, spleen, heart, lung, brain and kidney. This delivery technique uses the same intravenous route used for delivery of viral vector systems to murine neonates, via the superior temporal vein [5]. To investigate the possibility of liver-specific gene expression by this procedure, application of a pDNA driven by the human alpha-1-antitrypsin (AAT) promoter, previously shown to sustain strong levels of transgene expression in the liver *in vivo* for up to six months [6,7], results in transient gene transfer to hepatocytes for at least ten days. Non-viral gene transfer by this delivery procedure will undoubtedly be useful for other gene therapy studies investigating the performance of non-viral vectors in a unique physiological environment that is the developing mouse neonate.

2. Materials and methods

2.1. Vectors used in this study

The plasmids used in this study were pUbC-Luc-S/MAR, kindly provided by Dr Carsten Rudolph (University of Munich, Germany) and plasmid pLucA1, described in a previous paper [6] (Fig. S1). The plasmids were amplified in *E. coli* DH10B cells (Invitrogen Ltd., Paisley, UK) and isolated using an Endotoxin free Maxi prep (Qiagen, Crawley, UK). All restriction enzymes were purchased from NEB Biolabs (New England Biolabs, Hitchin, UK).

2.2. Animal studies

All animal care and experimental procedures complied with UK Home Office regulations. Female MF1 mice (Harlan, UK) were time-mated with MF1 male mice to obtain neonates of known gestational age. All neonatal injections were performed within the first 36–48 h of life. Polyplexes were formed according to the manufacturer's instructions. For the injection of a mouse pup with a polyplex at an N/P 8 ratio, 10 μg DNA was diluted in 5% isotonic glucose solution to a final volume of 15 μl and 1.6 μl of a 22 kDa cationic linear polyethylemine (PEI) (*in vivo*-JetPEI, Polyplus Transfection UK) was diluted in another aliquot of 5% isotonic glucose solution to a final volume of 15 μl. Diluted PEI was then added to diluted DNA, vortexed briefly and allowed to complex for 30 min prior to injection. The mother of each litter (singly housed) was removed from the cage. All material and equipment used were rubbed with the scent of the mother's cage. The pup was placed briefly on a bed of ice for anaesthesia. A light source was used to visualize the superior temporal vein and the 30 μl volume of PEI/DNA was administered slowly intravenously using a 33-gauge needle and syringe (Hamilton, UK). After injection, the mouse pup was warmed on a heated mat and thoroughly rubbed with bedding to prevent rejection by the mother. The mother was then reintroduced into the cage. For identification of injected animals from their uninjected littermates, the footpad of each injected pup was marked by subcutaneous injection of approximately 1 μl of colloidal carbon suspension immediately following vector administration. The animals were killed by inhalation of isofluorane.

2.3. Photon correlation spectroscopy

Polyplex sizes were measured by photon correlation spectroscopy on a *Coulter Delta* N4 PCS plus 440SX photon correlation spectrometer. After assembly, all polyplexes were diluted 1 in a 1000 with distilled water, a concentration allowing the count rate to stabilize between 4 and 6×10^4 counts per second. The calculated unimodal distribution was used as the determinant for average diameter size.

2.4. In vivo bioimaging

Animals were injected intraperitoneally with D-luciferin (15 mg/ml)(Gold Biotechnology, USA) at a volume of 10 μl per 1 g of animal weight prior to anaesthetisation by isofluorane and imaged for bioluminescence with the IVIS Imaging 50 Series. Bioluminescent imaging was performed in a light tight chamber on a temperature-controlled adjustable stage while isofluorane was administered by means of a gas manifold at a concentration of 0.5%. Images were acquired at a medium binning level (4–8) and a 20 cm field of view. Acquisition times were either 60 or 120 s depending on the intensity of luminescence. The Xenogen system reports bioluminescence as photons/s/cm^2/seradian in a 2.86 cm diameter region of interest encompassing the liver. The autofunction was used to define the minimum for the scale at each time point. This value was adjusted to 5% of the maximum in each case. Data was analysed using Living Image Software 2.50.

2.5. Cytokine level determinations

Blood was collected and pooled from pups at several time points after injection and centrifuged at 10000×*g* for 5 min to collect the supernatant. Serum TNFα, IL-12 and IFNγ concentrations were determined by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (eBioscience, Hatfield, UK). The amounts of cytokines were determined in 100 μl of ×5 diluted serum loaded in triplicate.

2.6. Hepatic enzyme level determination

Blood was collected and pooled from pups at days one, five and ten post injection and centrifuged at 10000×*g* for 5 min to collect the supernatant. Hepatic enzyme levels were determined at the Clinical Pathology Laboratory at the Medical Research Council Harwell (Oxford, UK).

2.7. Southern blot analysis

For DNA analysis, total liver DNA was extracted using a GenElute mammalian genomic DNA kit (Sigma-Aldrich Company Ltd., Gillingham, UK). The isolated DNA was quantified using a NanoDrop ND1-1000 spectrophotometer (Labtech International Ltd., Ringmer, UK). For Southern analysis, total liver DNA (15 μg) was digested with an appropriate restriction enzyme (StuI) that is a single cutter to all plasmids, separated on 0.8% agarose gels (20 V, 20 mA overnight) and blotted onto nylon membranes (Hybond XL, Amersham plc, Little Chalfont, UK). A 408 bp DNA fragment derived from the restriction digest of a segment of the kanamycin region, which is common to all plasmids, using enzyme AlwNI, was labelled with ^{32}P (Rad-Prime labelling kit, Invitrogen) and used as a probe. The hybridization was performed in Church buffer (0.25 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 1% BSA, 7% SDS) at 65 °C for 16 h.

For the replication-dependent restriction assay, 20 μg of total liver DNA was digested with StuI and further digested with DpnI, MboI or Sau3AI enzyme overnight. Agarose gel separation and Southern analysis were then performed as mentioned before.

2.8. PCR

Isolated liver DNA was quantified using a Nanodrop ND1000 Spectrophotometer and adjusted to a concentration of 10 ng/μl with distilled water. PCR was conducted in a thermocycler T3 (Biometra) with the following primers (Invitrogen): AAT promoter: forward 5′-ATGGAAAAACGCCAGCAACG-3′; reverse — 5′-AAGGTCACCCCAG-TTATCGGAG-3′; product size: 327 bp. Template DNA was initially denatured by heating at 95 °C for 5 min, followed by 30 cycles of