

Improved Method of Recombinant AAV2 Delivery for Systemic Targeted Gene Therapy

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A major hurdle in most current gene therapy modalities is the ability to transduce target tissues at very high efficiencies that ultimately lead to therapeutic levels of transgene expression. We have developed a novel method of recombinant adeno-associated virus 2 (rAAV) delivery that results in increased vector transduction efficiencies using microspheres reversibly conjugated to rAAV vectors. We hypothesize that conjugation to microspheres should result in a higher effective concentration of vector as well as longer relative exposure time of vector to target cells as it moves through the tissue vasculature. *In vitro* experiments demonstrate that the same level of transduction seen with free vector can be achieved using 1% of vector when conjugated to microspheres. In addition, using magnetic microspheres, the region of infection can be targeted. *In vivo*, we demonstrate that microsphere-mediated delivery of rAAV vector results in higher transduction efficiencies than delivery with free vector alone when administered either intramuscularly or intravenously. Furthermore, we demonstrate targeting of transgene expression to specific tissues by retention of microsphere-bound vector in the capillary bed. These studies demonstrate a novel method to deliver rAAV vectors more effectively that could prove to be a successful alternative mode of virus-mediated human gene therapy.

Key Words: dependovirus, gene therapy, vehicles, drug carriers, microspheres

INTRODUCTION

In recent years, viral vector-based human gene therapy approaches have been developed as potentially effective, alternative treatment modalities for a variety of diseases. In particular, adeno-associated virus 2 (AAV), a defective parvovirus of human origin, has been demonstrated to be one such promising vector. AAV is an ideal vector for viral-based human gene therapy because it has not been associated with any known pathology, and post-infection, the viral genome integrates into the human chromosome [1]. Currently, recombinant AAV (rAAV) vectors are being evaluated in human clinical trials for treatment of diseases such as cystic fibrosis and hemophilia B [2,3]. As with most gene therapy vectors, obstacles in the efficient use of rAAV vectors for a variety of disease models include sub-therapeutic levels of transduction and the ability to target the site of gene transfer.

Previously, adenovirus binding to target cells has been shown to be a function of adsorption time, particle concentration, and distance to target cell surface [4]. It is

probable that AAV is also affected by such circumstances. We have developed a system in which to overcome these limitations in the context of rAAV vectors for human gene therapy. To this end, we conjugate vector to microsphere carriers. Delivery by way of microspheres retards the flow of vector through the vasculature, thus resulting in increased exposure time of vector to target cells. In addition, this system results in increased localized multiplicity of infection (MOI), as the concentration of vector on each microsphere results in exposure to greater numbers of vector particles to each cell.

RESULTS

Recombinant AAV Reversibly Binds to Microspheres

The initial steps of AAV infection have become more clearly defined with the identification of heparan sulfate (HS) proteoglycan as a cellular receptor for AAV and human fibroblast growth factor receptor-1 and α V β 5 integrin as cellular co-receptors [5–7]. Since the identification of the cellular receptors for AAV, the use of column

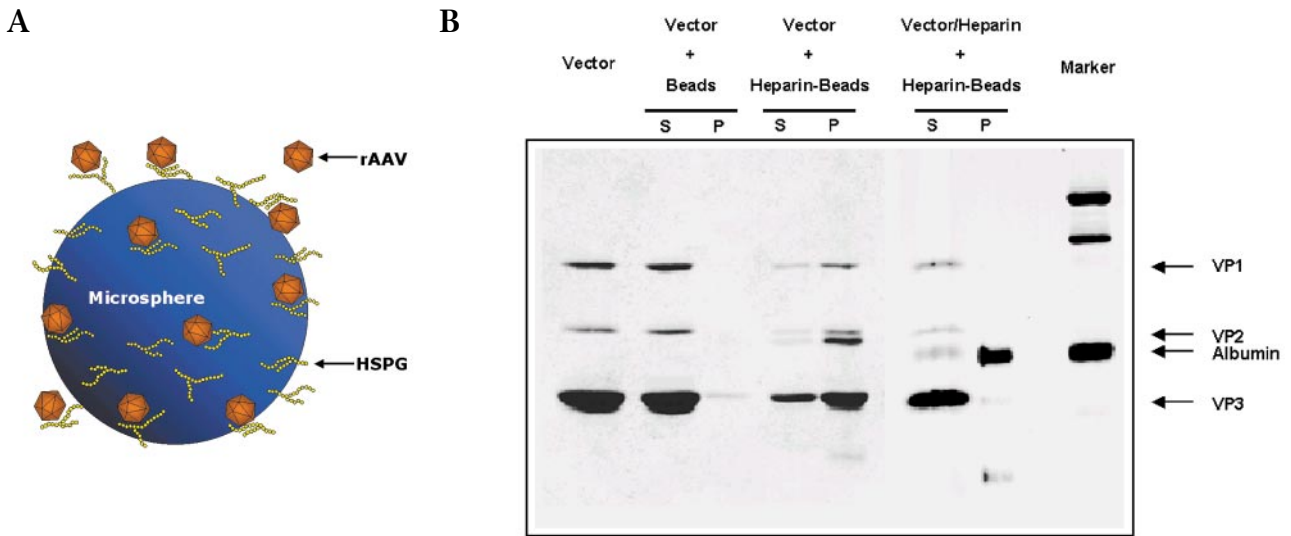


FIG. 1. Recombinant AAV reversibly binds microspheres. (A) Schematic model of microsphere-conjugated rAAV. (B) SDS-PAGE analysis of vector binding to microspheres. Recombinant AAV vector (lane 1) was incubated with polystyrene microspheres either uncoated (lanes 2 and 3) or coated with heparan sulfate (lanes 4 and 5) and in the presence of excess free heparin (lanes 6 and 7). After incubation, the samples were centrifuged and the resultant supernatant or pellet (microspheres) were analyzed by SDS-PAGE.

chromatography using heparin-conjugated supports has become widely established as an efficient step in rAAV vector purification schemes [8]. Taking advantage of this property, we proposed to conjugate rAAV to microsphere supports that are coated with HS molecules (Fig. 1A). Avidinylated microspheres were either coated or not coated with biotinylated HS and assayed for their ability to bind rAAV. The binding reaction solutions were subjected to centrifugation to separate the microspheres from the supernatant, and both fractions were analyzed by SDS-PAGE (Fig. 1B). In the absence of HS, rAAV vector remained free, or unbound in the supernatant, from the pelleted microspheres (lanes 2 and 3, Fig. 1B). When the microspheres were coated with the biotinylated HS, most of the vector bound to the microspheres (lanes 4 and 5, Fig. 1B). Competition for virus already bound to microspheres, by the addition of excess soluble HS to the complexes, clearly demonstrated that virus binding to the microspheres was reversible, as shown by the presence of vector in the supernatant (lanes 6 and 7, Fig. 1B).

Transduction Efficiency Is Increased Using Microsphere-Conjugated rAAV *in Vitro*

To assay the ability of the microsphere-conjugated vector to infect cells, *in vitro* infection experiments were performed. We infected C12S cells, derivative HeLa cells stably expressing the AAV Rep proteins [9], with unbound, rAAV encoding the gene for green fluorescent protein (GFP), rAAV bound to polystyrene microspheres, or rAAV bound to magnetic microspheres. Cells were analyzed for GFP expression by both fluorescence microscopy and fluorescence-activated cell sorting (FACS) 48 hours after

infection (Fig. 2). A significantly higher percentage of GFP-positive cells were seen in those cells infected with microsphere-conjugated vector than with free vector (Figs. 2A–2C). The region of transduction could be targeted *in vitro* using the magnetic microsphere-conjugated vector and a small square-shaped magnet to target the microspheres (Fig. 2D). This may have implications for directing vector to target cells. FACS analysis of cells infected *in vitro* demonstrated the improved transduction efficiency of the microsphere-conjugated vector (Fig. 2B). The transduction efficiency of cells infected with vector conjugated to polystyrene microspheres increased to $58.9 \pm 2.57\%$ from $8.48 \pm 5.77\%$, and even greater transduction was seen with vector bound to magnetic microspheres, infecting $75.84 \pm 2.75\%$ of cells. It was also noted that 1% of vector, when conjugated to the magnetic microspheres, resulted in the same levels of transduction achieved with free vector alone.

Transduction Efficiency Is Increased Using Microsphere-Conjugated rAAV *in Vivo*

We had previously noted that after intramuscular (IM) injection of a tracing dye, the dye was cleared from the muscle tissue. We hypothesized that delivery of vector conjugated to the microspheres would result in increased dwell-time of the vector within the muscle tissue. As significantly higher transduction efficiencies were seen from *in vitro* experiments using the microsphere bound vector, we decided to study the effects *in vivo*. We injected, intramuscularly, 1.75×10^8 infectious particles of either microsphere-bound or free rAAV encoding the CMVp-driven β -galactosidase (*lacZ*) gene into the right gastrocnemius of

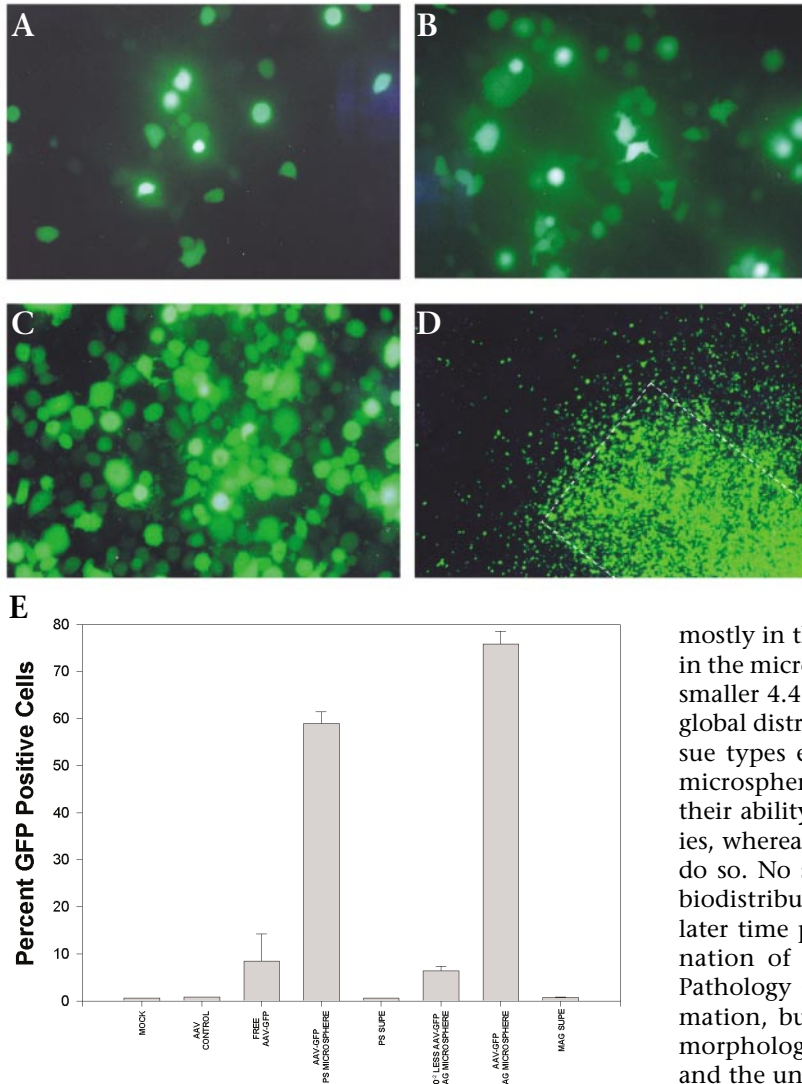


FIG. 2. *In vitro* transduction of vector into C12S cells. C12S cells were infected with free rAAV encoding GFP (A), polystyrene microsphere conjugated-rAAV encoding GFP (B), or magnetic microsphere conjugated-rAAV encoding GFP (C, D). Cells shown in (C) and (D) were infected in the presence of a magnet (magnet placement indicated by white dotted line). GFP expression was assayed by fluorescence microscopy. (E) FACS analysis of *in vitro* transduction using microsphere-conjugated vector. C12S cells were either mock infected or infected with control (non-GFP encoding), polystyrene microsphere conjugated-, or magnetic microsphere conjugated-rAAV. GFP expression was analyzed by FACS analysis, 48 hours after infection.

and microspheres were visualized by fluorescence microscopy (Fig. 4). The largest microspheres used, which were approximately 10 μm in diameter, targeted different organs, and targeting was dependent on the route of administration. IV administration resulted in the microspheres depositing

mostly in the lungs, whereas portal vein delivery resulted in the microspheres remaining in the liver. Delivery of the smaller 4.4 μm diameter microspheres resulted in a more global distribution, as they were detected in almost all tissue types examined. The widespread deposition of the microspheres may be attributed to their small size and their ability to pass through the majority of the capillaries, whereas the 10 μm microspheres may be too large to do so. No significant difference was seen in the relative biodistribution of the microspheres when examined at later time points (data not shown). Independent examination of all the tissues by the University of Florida Pathology Core revealed some minor regions of inflammation, but no clinically significant immunological or morphological differences between the treated animals and the uninjected animals.

As the regions of microsphere deposition *in vivo* could be limited by the size of the microspheres and the route of delivery, we investigated whether or not the sites of expression of the therapeutic gene from microsphere-conjugated vector correlated with the sites of microsphere deposition *in vivo*. For these studies, we used a rAAV vector expressing gene (*SERPINA1*) encoding the human α -1-antitrypsin (AAT) driven by the hybrid cytomegalovirus immediate-early enhancer chicken β -actin (CB) promoter. The AAV-CB-*SERPINA1* vector has been previously shown to successfully transduce mouse muscle and liver tissues *in vivo* and a human cystic fibrosis epithelial cell line *in vitro*, and has demonstrated significant potential as an alternate gene therapy vector for the treatment of AAT deficiency [11,12]. AAT is a serine protease inhibitor that protects lung elastase from protease and neutrophil elastase-mediated degradation. Patients deficient in human AAT can develop pan-acinar emphysema. For this study, we conjugated 1×10^9

129/SvJ mice. Tissues were assayed for β -galactosidase activity 4 weeks after injection, using the Galacto-Star β -galactosidase detection assay [10]. β -Galactosidase enzyme activity in animals treated with microsphere-conjugated vector was approximately double that of animals treated with unbound vector (Fig. 3).

Route of Delivery and Microsphere Size Affect Tissue Targeting

In addition to using microsphere supports to increase transduction efficiencies using rAAV2 vectors, we also investigated the possibility of using the microspheres to target the regions of infection *in vivo*. To examine the regions of microsphere deposition *in vivo*, fluorescent microspheres were delivered into BALB/cJ mice either by intravenous (IV) administration into the tail vein or by direct injection into the portal vein. Tissues were sectioned

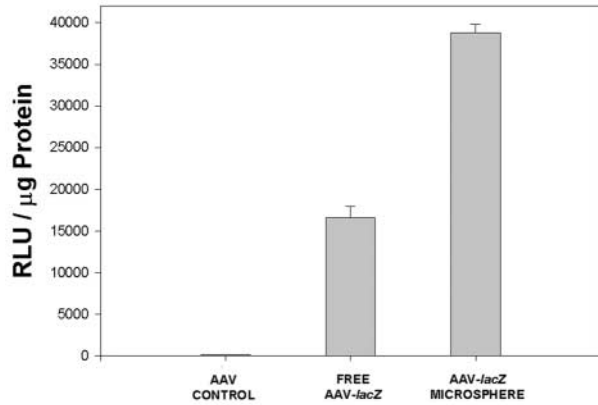


FIG. 3. Enzymatic analysis of *in vivo* transduction using microsphere-conjugated vector. 129/SvJ mice were injected IM with either free vector or microsphere-conjugated rAAV encoding for *lacZ*. Tissue homogenates were assayed for β -galactosidase expression using the Galacto-Star β -galactosidase detection assay 4 weeks post-injection.

infectious particles of AAV-CB-*SERPINA1* to 10 μm microspheres. Free vector and microsphere-bound vector was then administered intravenously into the tail vein. To assay generalized transduction levels, serum levels of human AAT were measured by ELISA (Fig. 5A). The serum levels of human AAT were higher in the animals treated with microsphere-conjugated vector as compared to levels seen in animals treated with free vector 2 weeks after injection, and this increase was maintained 10 weeks later. During that ten-week period after injection, maximal level of serum human AAT from microsphere-mediated rAAV delivery was 1182.9 ng/mL; this is approximately five times greater than the average maximal level of 247.2 ng/mL seen in the animals treated with free vector. More importantly, we examined the regions of transduction with respect to microsphere deposition *in vivo*. Based on the data from the distribution study using the fluorescent microspheres, we expected that the AAV-CB-*SERPINA1* coated microspheres would deposit mostly in the lungs, because of the size of the microspheres used as well as the route of vector delivery (Fig. 4). Immunostaining of tissues using an anti-human AAT antibody clearly demonstrated that regions of transduction were re-directed toward tissues in which microsphere deposition occurred (Figs. 5B and 5C). Administration of vector conjugated to 10 μm microspheres resulted in significant transduction of lung tissue when delivered intravenously into the tail vein. Conversely, there was no evidence of transgene expression in the lung in animals administered free vector. Negative control tissues from animals in which microsphere-bound rAAV encoding a CB promoter-driven *GFP* gene was delivered intravenously into the tail vein did not result in any immunostaining using the anti-human AAT antibody (data not shown). Although numerous regions of transduction were noted throughout the entire tissue,

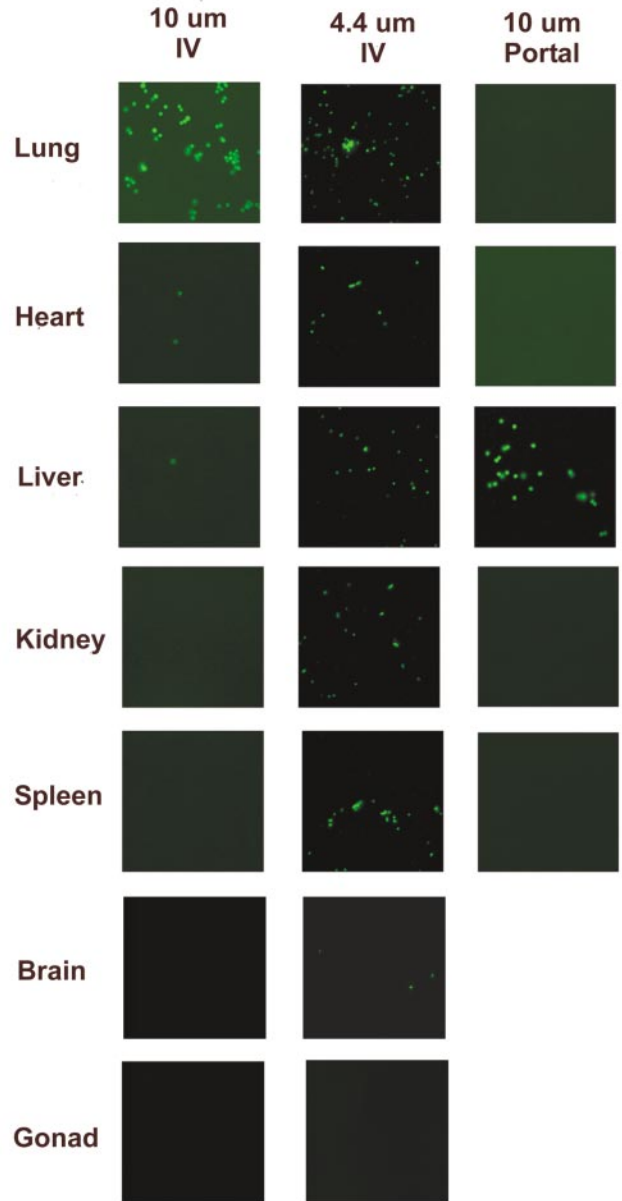


FIG. 4. Distribution of microspheres *in vivo*. Fluorescent microspheres of varying diameters were injected intravenously, either into the tail vein or into the portal vein in male BALB/cJ mice. Serial 15 μm frozen sections were made and visualized by fluorescence microscopy. Representative sections are shown.

those regions were not in the immediate vicinity of the deposited microspheres. It is possible that, as the microspheres traversed through the tissue vasculature, vector particles bound to and infected cells that it passed, and that the deposition patterns we see in the tissue sections reflect the terminal resting points of the microspheres. Much of the transduction occurred in the lung arterioles and may also correspond to cell types that express high levels of the cellular receptors for AAV2.

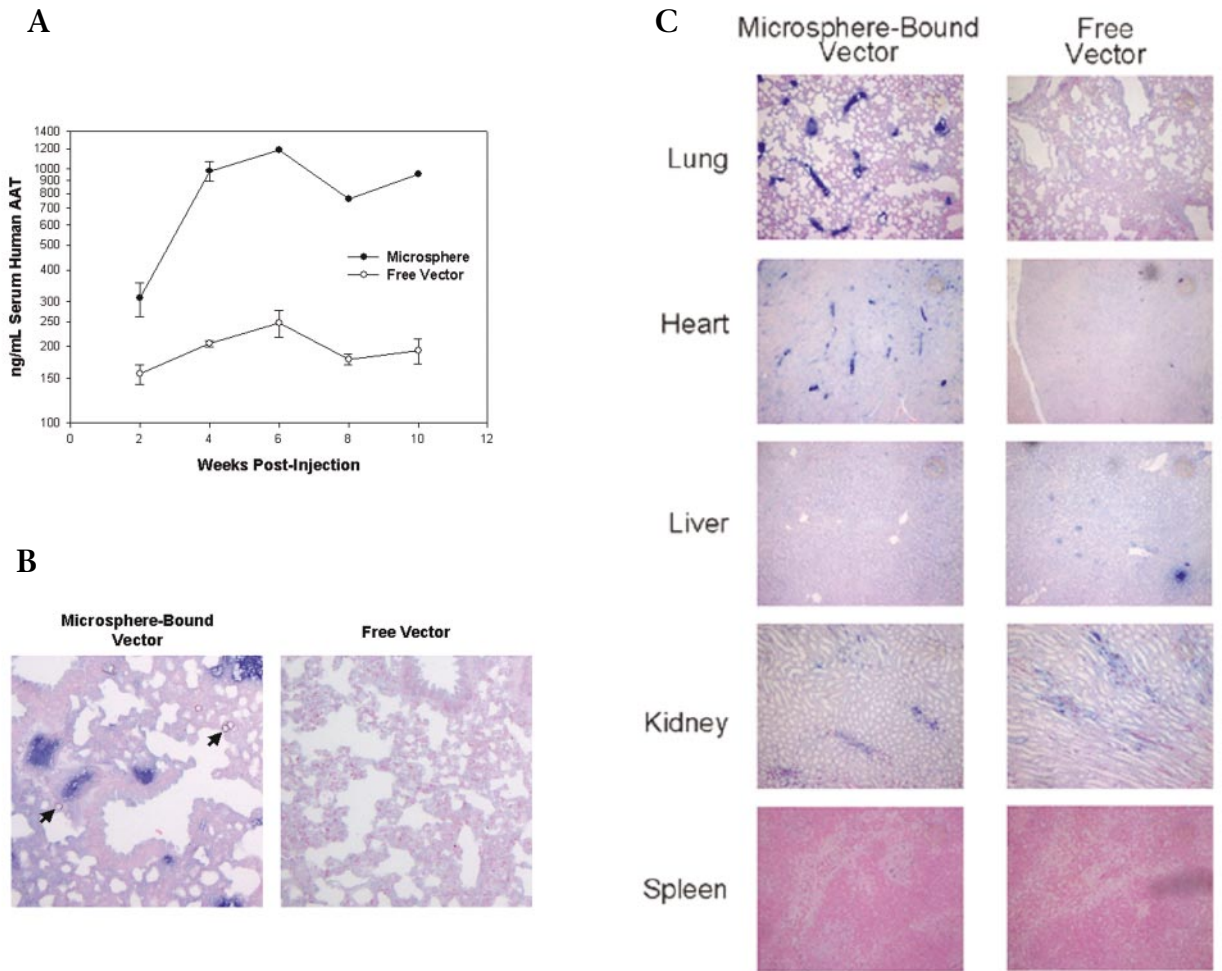


FIG. 5. Systemic delivery of microsphere-conjugated AAV-CB-*SERPINA1* *in vivo*. (A) Serum levels of human AAT as determined by ELISA. (B) Tissue immunostaining for human AAT ($\times 100$). Positive staining results in a blue precipitate. (C) Lung tissue from animals treated with either free or microsphere-bound vectors, $\times 200$. The arrowheads indicate microspheres deposited in lung tissue.

DISCUSSION

The ability of rAAV to transduce cells is limited by a variety of factors such as cellular receptor density, the MOI, and the time of exposure to vector particles. Much insight into rAAV-mediated transduction had been gained with the identification of the cellular receptors for rAAV. In addition, recent works describing the modes of entry into target cells [13–16], structural status of the rAAV genomes [17–20], the cell-cycle status of the infected cell [21,22], and the involvement of intracellular proteins in rAAV-mediated transgene expression [14,23–25] have provided a better understanding of the circumstances that factor into the ability of rAAV to transduce cells. Each facet governing rAAV transduction can be manipulated to enhance the abilities of rAAV as a vector for human gene therapy. For example, the use of strong tissue-specific or inducible promoters or the

inclusion of novel cis-acting elements in the vector has resulted in significant increases in transgene expression [26,27]. Other examples include the generation of alternate serotype vectors [28–30] or *cap* mutations that incorporate targeting peptide moieties into the viral capsid protein to enhance transduction of target cells [31–34], or the treatment of cells with a variety of pharmacologic compounds which render cells more permissive for rAAV-mediated transgene expression [25,35–38].

In this work, we have demonstrated an improved method for the physical delivery of rAAV vectors *in vivo* in which virion particles are conjugated to microsphere supports. Previous studies that are similar in concept have demonstrated the successful delivery of therapeutic compounds such as chemotherapeutic agents, synthetic hormones, and genetic elements using a microsphere-mediated strategy, some of which have been approved for use in humans [39–43]. In addition, work

by Kalyanasundaram *et al.* [44] demonstrated the ability to encapsulate a biologically active recombinant adenoviral vector that could be released in a time dependent manner. Taken together, these studies have demonstrated the ability to deliver and release therapeutic agents using polymer matrix microparticles. In our study, we demonstrated that delivery of microsphere-conjugated rAAV vectors results in both higher levels of generalized transduction and in the ability to re-direct the location of transduction events both *in vitro* and *in vivo*, as compared to delivery of free vector. This method likely resulted in longer periods of exposure of target cells to vector with higher localized effective concentrations of vector particles, ultimately resulting in higher transduction efficiencies. Other studies have also shown that increasing exposure time to gene transfer vectors results in increased levels of transduction efficiency [45–49]. The addition of a magnetic moiety to these microspheres may add yet another mode in which to retain the microsphere-vector conjugates within the desired tissue beds *in vivo* [40,43]. As there exists the potential for microspheres to remain within target tissues permanently, which could lead to undesirable or untoward effects, we are currently designing biodegradable microspheres. Although no significant immunological or inflammatory responses have been noted in the histological examination of the tissues examined thus far (data not shown), it will be important to thoroughly examine the immunological impact the microsphere-vector conjugates will have *in vivo*. Continuing studies should further demonstrate the utility of microsphere-conjugated vectors and their applications in human gene therapy approaches *in vivo*.

MATERIALS AND METHODS

Cells and viruses. Adenovirus-transformed human embryonic kidney cells, 293, were acquired from the American Type Culture Collection (ATCC; Manassas, VA). The C12S cell line was provided by Phillip Johnson (The Ohio State University, Columbus, OH) and have been described [9]. Monolayer cells were maintained at 37°C, 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The recombinant AAV plasmid, UF5 (pCMVp-GFP), was provided by Sergei Zolotukhin (University of Florida Powell Gene Therapy Center, Gainesville, FL) and has been described [50]. The recombinant AAV plasmids pCMVp-lacZ and pCBp-GFP were generated by standard cloning methods. Recombinant AAV vectors were generated, purified, and titered at the University of Florida Vector Core Lab as described [8].

Conjugation of rAAV to microspheres. To block nonspecific binding, 100 μL avidinylated polystyrene or magnetite microspheres (Bangs Laboratories, Indianapolis, IN) were washed three times with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Excess BSA was removed by washing three times in PBS. Microspheres were resuspended in 100 μg/mL biotinylated HS (Celsus Laboratories Inc., Cincinnati, OH) and incubated at room temperature for 1 hour. Unbound HS was removed by washing in PBS three times. Recombinant AAV was allowed to bind to the microspheres for 1 hour at room temperature. Unbound vector was removed by washing three times in PBS. Vector-bead conjugates were resuspended in PBS.

Analysis of vector-microsphere conjugates. Recombinant AAV vector was bound to avidinylated polystyrene microspheres in the presence or absence

of biotinylated HS. Excess non-biotinylated HS (Sigma, St. Louis, MO) was used in competition studies. Post conjugation, the microspheres were pelleted by centrifugation and both the pellet and the supernatant were analyzed by the Laemmli gel method on a 10% SDS-polyacrylamide gel. Viral capsid proteins were visualized using the diamine method of silver staining [8].

***In vitro* infections.** *In vitro* infections were performed by seeding approximately 1×10^5 C12S cells in 12-well dishes and allowing them to adhere for 24 hours at 37°C, with 5% CO₂. Infections were then performed in the maximal volume (3 mL) of DMEM allowed in the culture well. Unbound or microsphere-bound vector encoding GFP driven by the cytomegalovirus immediate-early (CMV) promoter was added gently to the top of the medium and allowed to infect for 15 minutes, after which cells were washed with PBS three times. Medium containing 500 pfu/mL wild-type adenovirus type 5 was added to the cells and incubated for 48 hours at 37°C, with 5% CO₂. Expression of GFP was detected either by fluorescence microscopy or by FACS analysis using a FACSort flow cytometer (Becton-Dickinson Biosciences, Franklin Lakes, NJ) at the University of Florida Interdisciplinary Center for Biotechnology Research.

Animal studies. Animals were treated according to the National Institutes of Health guidelines for animal care and the University of Florida Institutional Animal Care and Use Committee guidelines. Adult 129/SvJ mice were administered 1.75×10^8 AAV-CMVp-lacZ, either free or conjugated to microspheres, by direct injection into the right gastrocnemius. The gastrocnemius was isolated and frozen in OCT cryoembedding compound 4 weeks after injection. Frozen sections (15 μm) of the entire tissue were pooled into a crude tissue homogenate and assayed for enzyme activity using the Galacto-Star chemiluminescent reporter gene assay system (Tropix Inc., Bedford, MA) [10].

For the tissue targeting studies, adult male BALB/cJ mice were administered 100 μL of a 1% solids solution of fluorescently labeled microspheres of varying sizes (Spherotech Inc., Libertyville, IL). Microspheres were delivered by either intraportal or intravenous infusion. Tissues were isolated and frozen in OCT cryoembedding compound 5 or 60 minutes after infusion. Frozen sections (15 mm) were visualized by fluorescence microscopy. Representative sections are shown. For examination of tissue pathology, microspheres were delivered. All tissues that were examined for biodistribution were harvested and processed by the University of Florida Pathology Core 5 days after administration. Tissues were briefly fixed in 10% formalin and embedded in paraffin. Sections were analyzed by hematoxylin and eosin staining and independently reviewed in the University of Florida Pathology Core.

The *in vivo* systemic delivery of a therapeutic vector was performed by administering 129/SvJ mice with 1×10^9 infectious particles of AAV-CB-SERPINA1 intravenously by way of the tail vein, either unconjugated or conjugated to 10 μm polystyrene microspheres. Serial serum samples were assayed for human AAT by ELISA as described ($n = 3$ for free vector group for weeks 0–10 and microsphere group for weeks 0–4, $n = 1$ for microsphere group weeks 6–10) [11,12]. Processing and immunostaining of tissues was performed by the University of Florida Pathology Core. Tissues were fixed in 10% formalin and embedded in paraffin. Tissues were stained with a rabbit anti-human AAT primary antibody (Roche Molecular Biochemicals, Indianapolis, IN) and detection was carried out using the True Blue peroxidase system (Kirkegaard and Perry Laboratories, Gaithersburg, MD) as described [51]. Representative sections are shown. All photographs were taken using a Zeiss Axioskop, Olympus digital camera, and MagnaFire digital imaging software.

ACKNOWLEDGMENTS

We thank Marda Jorgensen (University of Florida), Martha Campbell-Thompson (University of Florida), and the University of Florida Pathology Core for their assistance. This work was partially supported by the Whitaker Foundation, NIH-NIDDK P01 DK58327, NIH-NIDDK R01 DK58236, NIH grants HL51811, HL59412, and RR00082.

RECEIVED FOR PUBLICATION DECEMBER 19, 2001;
ACCEPTED APRIL 23, 2002.

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