Targeted Gene Delivery to Vascular Tissue In Vivo by Tropism-Modified Adeno-Associated Virus Vectors

Stephen J. White, DPhil*; Stuart A. Nicklin, PhD*; Hildegard Büning, PhD; M. Julia Brosnan, PhD; Kristen Leike; Emmanuel D. Papadakis, BSc; Michael Hallek, MD; Andrew H. Baker, PhD

**Background**—Gene therapy offers an unprecedented opportunity to treat diverse pathologies. Adeno-associated virus (AAV) is a promising gene delivery vector for cardiovascular disease. However, AAV transduces the liver after systemic administration, reducing its usefulness for therapies targeted at other sites. Because vascular endothelial cells (ECs) are in contact with the bloodstream and are heterogeneous between organs, they represent an ideal target for site-specific delivery of biological agents.

**Methods and Results**—We isolated human venous EC-targeting peptides by phage display and genetically incorporated them into AAV capsids after amino acid 587. Peptide-modified AAVs transduced venous (but not arterial) ECs in vitro, whereas hepatocyte transduction was significantly lower than with native AAV. Intravenous infusion of engineered AAVs into mice produced reduced vector accumulation in liver measured 1 hour and 28 days after injection and delayed blood clearance rates compared with native AAV. Peptide-modified AAVs produced enhanced uptake of virions in the vena cava with selective transgene expression. Retargeting was dose dependent, and coinfusion of either heparin or free competing peptides indicated that uptake was principally independent of native AAV tropism and mediated via the peptide.

**Conclusions**—AAV tropism can be genetically engineered by use of phage display–derived peptides to generate vectors that are selective for the vasculature. (Circulation. 2004;109:513-519.)

**Key Words:** gene therapy ■ endothelium ■ viruses ■ peptides

Cardiovascular gene therapy is becoming a realistic clinical goal, as results in clinical trials for vein graft failure and stable angina prove. A critical feature of efficient gene delivery is target cell accessibility in vivo. Although certain surgical procedures (eg, bypass grafting) allow access, many sites are inaccessible (eg, pulmonary or renal tissue) but would be amenable to systemic delivery if appropriate vectors were available. Endothelial cells (ECs), which are in continuous contact with the bloodstream and integrally involved in cardiovascular abnormalities, are appropriate targets. The concept of EC targeting originates from studies that defined in vivo heterogeneity, such that many EC surface receptors seem to be unique to individual organs. Uptake of basic gene transfer vectors, including adenovirus, AAV, and lentivirus, by ECs after systemic delivery is poor, because the liver rapidly sequesters virus; thus, only therapies that take advantage of this natural tropism are feasible.

Adeno-associated virus (AAV) is a promising gene transfer vector for cardiovascular therapies. AAV tropism is dictated by distribution of its primary cell-tethering receptor heparan sulfate proteoglycan (HSPG) and internalizing the coreceptors fibroblast growth factor receptor 1 and αβ integrin. Although AAV transduces ECs, the efficiency is lower relative to hepatocytes. Modifying AAV to remove native tropism in combination with provision of target cell–specific tropism (retargeting) is a prerequisite for development of systemically injectable vector platforms. New tropism may be supplied through antibodies, ligands, or small targeting peptides (reviewed by Nicklin and Baker). A defined site in the capsid of AAV serotype 2 (AAV-2) has been identified as an efficient site for insertion of targeting peptides. Here, we have isolated 2 novel venous EC-selective targeting peptides that redefine AAV infectivity profiles in vitro and in vivo.

**Methods**

**Cell Culture**

Human umbilical vein ECs (HUVECs), human coronary artery ECs, and primary human hepatocytes were purchased from TCS (Botolph...
Peptide Sequences From Phage Display

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All peptides and their frequencies of isolation are indicated. Candidate consensus motifs are indicated by underlining. and peptides in bold represent the most frequently identified peptides.

Claydon). Human saphenous vein ECs were prepared as described previously.14 ECs were cultured in complete endothelium media (TCS), 20% (vol/vol) FCS, and antibiotics. HepG2 hepatocytes and the murine venous EC lines IP-1B and SVEC-4-1015 were maintained in minimal essential medium, 10% (vol/vol) FCS, and antibiotics. Peripheral blood mononuclear cells were isolated from whole blood by use of Ficoll Paque (Pharmacia Biotech).

Phage Display

Libraries (12-mer, New England BioLabs) were panned on 2 successive cultures of VSMCs, HepG2, and peripheral blood mononuclear cells (preclearing steps), then on HUVECs. Weakly associated phages were removed, high-affinity phages isolated by lysis in 30 mmol/L Tris/10 mmol/L EDTA (pH 8.0), and individual plaques sequenced after 5 rounds. Peptide homology was performed via BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Recovery studies for homogenous phages were as described previously.16 Briefly, 107 phages were incubated with cells for 1 hour at 4°C, washed 5 times, lysed in hypertonic buffer (30 mmol/L Tris/10 mmol/L EDTA), and quantified by titration.

Production of AAV Vectors

pRCmtp and pRCmsl were constructed with oligonucleotides encoding MTSLIPTPSNEANL and MSLIPTPPAVARP (peptide sequence italicized), annealed into the Miel-Asc I site of pRC-99 after position 587 in the AAV-2 capsid, and produced as described previously.13 Viruses express lacZ from the cytomegalovirus promoter. Genomic particle (GP) titers were quantified by dot blot and real-time polymerase chain reaction (PCR). For hepatic colony assays, AAV was loaded onto columns with heparin immobilized on cross-linked 4% agarose beads (Sigma). The flow-through, 3 column washes (PBS, 1 mmol/L MgCl2, 2.5 mmol/L KCI), and elution fractions (PBS containing 1 mmol/L MgCl2, 2.5 mmol/L KCI, and 1 mol/L NaCl) were analyzed by dot blot.

In Vitro Cell Transduction Assays

AAV transduction was quantified at 5000, 10,000, or 20,000 GPs/cell in the presence of helper Ad (multiplicity of infection, 10). Cells were exposed to virus for 16 hours, the media were changed, and the cells were subsequently incubated until 72 hours. Cells were lysed, and β-galactosidase expression was quantified by use of Galactolight Plus (Tropix).

Virus Distribution Studies In Vivo

A total of 1.5 × 1010 or 3 × 1011 GPs of AAVwt, AAVmtp, or AAVmsl were infused intravenously into male 5-week-old BALB/C mice before euthanasia. Organs were removed and snap frozen, and DNA was extracted (Qiagen) and quantified by use of PicoGreen (Cambridge Biosciences). A lacZ quantification standard curve was generated from serial dilutions of each AAV preparation by use of FastStart DNA Master SYBR Green I (Roche) with 0.5 mmol/L forward (5’-ATCAGACCACGCAAATGGA-3’) and reverse (5’-CATCAAGCGTTGATCTCGC-3’) lacZ primers. Total DNA (20 ng) from a defined tissue mass was amplified, and products were quantified by melting curve analysis at crossover points using the Lightcycler Data Analysis software (Roche). Results were expressed as genomic AAV particles/mg.
tissue. The following reaction conditions were used: denaturation, 95°C, 600 seconds; amplification, 95°C, 3 seconds; annealing, 60°C, 10 seconds; elongation, 72°C, 20 seconds; and data collection, 89°C, 1 second (30 to 50 cycles). Products were analyzed by melting-curve analysis after initial denaturation at 95°C with cooling at 20°C/s to 45°C, incubation for 15 seconds, and termination at 99°C. For competition experiments, AAVs were coinfused with either 100 IU heparin or 0.5 mg free C-terminally amidated peptide (MSLTTPPAVARPGGS or MTPFPTSNEANLGGGS). DNA was extracted, quantified, and subjected to quantitative PCR as described above.

**Analysis of Transgene Expression**

β-Galactosidase activity was measured in tissue homogenates with the Galactolight Plus kit (Tropix). Protein concentration was quantified with the microBCA assay (Pierce). Detection of β-galactosidase by immunohistochemistry was performed on paraformaldehyde-fixed, wax-embedded sections with rabbit anti–β-galactosidase antibody (ICN Biomedicals) 1/1200 or matched rabbit IgG nonimmune control, followed by detection with biotinylated goat anti-rabbit secondary antibody (1/200), ABC kit (Vector Laboratories) and Sigma Fast diaminobenzidine staining tablets (Sigma). Sections were counterstained with hematoxylin for 5 seconds.

**Statistical Analysis**

Data were analyzed by ANOVA for multiple comparisons or unpaired Student’s t test and are shown as mean±SEM. Data were considered significant at a value of P<0.05.

**Results**

**Identification of Targeting Peptides**

First, we sought to identify novel EC-targeting peptides by phage display. Using a selective in vitro biopanning procedure (see Methods), we identified 49 individual peptides (Table), many of which were isolated between 2 and 10 times.

Peptides MSLTTPPAVARP and MTPFPTSNEANL were repeated 10 and 6 times, respectively. A number of consensus motifs were identified (Table). No homology to human sequences was identified by database searches. Phage recovery studies on HUVECs, VSMCs, and hepatocytes with 23 of the selected peptides demonstrated EC-selectivity (not shown except MSLTTPPAVARPGGS or MTPFPTSNEANLGGGS). DNA was extracted, quantified, and subjected to quantitative PCR as described above.

**Characterization of Peptide-Modified AAV Vectors In Vitro**

We genetically inserted MSLTTPPAVARP or MTPFPTSNEANL peptides into AAV-2 after residue 587 and created AAVmsl and AAVmtp with titers comparable to those of wild-type AAV (AAVwt) (not shown). In HUVECs (Figure 2A), transduction for each AAV was dose dependent, and both AAVmsl and AAVmtp produced significantly lower transduction than AAVwt (Figure 2B). We compared heparin dependence for each AAV in ECs (Figure 2C). Transduction with AAVwt and AAVmsl in presence of competing heparin (1 IU/5×10⁶ GPs AAV), D, Heparin column binding assay. See Methods for details. Flow-through, column washes, and elution fractions (1 mol/L NaCl) were analyzed by dot blot.

**Figure 2.** Analysis of AAV transduction in vitro. A, HUVECs were infected with 10 000 and 20 000 GPs/cell of either AAVwt, AAVmsl, or AAVmtp, and β-galactosidase was quantified at 72 hours. B, Transduction of HepG2 hepatocytes at 5000 and 10 000 GPs/cell with analysis as for ECs. C, Transduction of HUVECs at 10 000 GPs/cell by AAVwt, AAVmtp, and AAVmsl in presence of competing heparin (1 IU/5×10⁶ GPs AAV). D, Heparin column binding assay. See Methods for details. Flow-through, column washes, and elution fractions (1 mol/L NaCl) were analyzed by dot blot.
was significantly inhibited by heparin (not shown). AAVmtp produced negligible HepG2 transduction that was inhibited with heparin, demonstrating that it represented residual native tropism (not shown). Infectivity profiles and heparin dependence for each virus in primary human hepatocytes was essentially the same as in HepG2 cells, although the absolute levels of transgene expression were lower (not shown). A direct comparison of transduction levels in HepG2 hepatocytes and primary human hepatocytes at 10,000 GPs/cell revealed that AAVwt produced 200 μg β-galactosidase/mg protein in HepG2 hepatocytes but 8 μg β-galactosidase/mg protein in primary hepatocytes. Both peptide modifications significantly reduced the efficiency of AAV transduction into primary hepatocytes, resulting in an improvement in the primary hepatocyte-to-primary EC ratio of 4:1 for AAVwt to 1:1 for both AAVmsl and AAVmtp. Heparin dependence was also assessed by use of heparin columns (Figure 2D). In contrast to AAVwt (found exclusively in high-salt eluate), substantial proportions of AAVmsl and AAVmtp were detected in the column wash, consistent with the cell-transduction studies (Figure 2C). Importantly, both peptide-modified AAVs showed reduced transduction in primary human coronary ECs, demonstrating their venous selectivity (not shown). Because of the cross-species targeting we observed (Figure 1B), we also assessed transduction of each AAV in the murine venous EC line IP-1B. Each peptide-modified AAV produced efficient transduction, and AAVmtp was not significantly different from AAVwt (not shown). Assessment of the transduction of human VSMCs confirmed that each peptide-modified vector transduced venous ECs selectively and not other vascular cell types (not shown).

**Characterization of Peptide-Modified AAV Vectors In Vivo**

We infused 1.5×10^{10} GPs of each AAV into BALB/c mice and assessed particle biodistribution by real-time PCR 1 hour after infusion. Real-time PCR allows tracking of virions to specific sites in vivo without other confounding components of AAV transduction, eg, trafficking and nuclear gene conversion, which vary widely in different tissues but occur independently of cell-surface binding. AAVwt accumulated predominantly in the liver (Figure 3A). Both peptide insertions significantly reduced liver virion accumulation (Figure 3A). Likewise, lung and kidney accumulation (Figure 3A) was significantly lower for AAVmtp and AAVmsl compared with AAVwt, whereas brain (Figure 3A) and heart (Figure 3A) were not different from AAVwt. Because reduced virion accumulation was evident for both AAVmsl and AAVmtp, we quantified virions present in blood 30, 60, and 180 minutes after infusion (Figure 3B). Interestingly, both mutants had delayed clearance from the blood at early time points compared with AAVwt. No differences were observed at 180 minutes. We next analyzed arterial and venous blood vessels (Figure 4). Uptake in the aorta was significantly lower for AAVmsl and AAVmtp than AAVwt (Figure 4A). In contrast, AAVmsl levels in the vena cava were similar to AAVwt (Figure 4B). Importantly, AAVmtp demonstrated significantly higher accumulation than AAVwt (Figure 4B), indicating that the venous selectivity observed by in vitro studies was replicated in vivo. Next, we assessed the specificity of the targeting to vena cava using competition assays at 1 hour (Figure 4). Coinfusing heparin with AAVs significantly blocked AAVwt but not AAVmsl or AAVmtp in the vena cava at 1 hour (Figure 4C). For both AAVmsl and AAVmtp, coinfusion of the respective free peptide in vivo significantly reduced accumulation in venous tissue (Figure 4D).

We next analyzed AAV distribution 28 days after infusion (Figure 5). Reduced AAV accumulation in the liver for AAVmsl and AAVmtp was confirmed by real-time PCR (Figure 5A). Similar reductions were observed in spleen and lung (Figure 5A). We analyzed AAVwt and AAVmtp further at a high dose (3×10^{11} GPs/mouse) and observed that the reduced virion accumulation was dose dependent (Figure 5B). At this dose, AAVmtp produced 29.7±9.7% and AAVmtp, 2.9±0.45% (n=4 in all groups) of AAVwt values. Importantly, in the vena cava at low dose (1.5×10^{10} GPs/mouse), AAVmsl and AAVmtp were readily detected by real-time PCR, but AAVwt was undetectable in all animals tested (Figure 5C). Again, this was dose dependent when AAVwt and AAVmtp were analyzed at the higher dose (Figure 5C). We compared the mean vena cava-to-liver ratios

![Figure 3. Biodistribution in vivo using modified AAV vectors. A, 1.5×10^{10} GPs of AAVwt, AAVmtp, and AAVmsl or PBS were infused into BALB/c mice, and particle distribution was quantified by real-time PCR in all major organs. *P<0.05 vs AAVwt (n=6 mice/group). B, Circulating AAVwt, AAVmsl, and AAVmtp were quantified at 30, 60, and 180 minutes after infusion of 1.5×10^{10} GPs using real-time PCR (n=3 mice/group).](http://circ.ahajournals.org/)
at 1 hour, and they were 0.1 for AAVwt and 1.3 for AAVmtp. At 28 days after injection of $3 \times 10^{11}$ GPs, AAVwt produced a mean vena cava-to-liver ratio of 0.016, and for AAVmtp, it was 6.5. Thus, there was a 400-fold improvement in vena cava-to-liver gene delivery with AAVmtp. To further define selectivity, we analyzed aortas at 28 days after infection, and both AAVmsl and AAVmtp produced reduced accumulation compared with AAVwt (not shown).

Ultimately, transgene expression is the most important parameter. We therefore analyzed β-galactosidase transgene expression at 28 days after infusion in both AAVwt- and AAVmtp-infected animals at $3 \times 10^{11}$ GPs/mouse. In accordance with real-time PCR data, transgene expression in liver homogenates was significantly reduced for AAVmtp compared with AAVwt (Figure 6A). Transgene levels in tissue homogenates from other organs were not above background levels for both AAVmsl and AAVmtp (not shown). Immunohistochemical analysis of the vena cava revealed selective expression of transgene in cells at the luminal surface of AAVmtp- but not AAVwt- or PBS-treated animals (Figure 6B). β-Galactosidase activity was not detected in the aortic tissue from animals treated with either AAV (not shown).

**Discussion**

The concept of developing systemically injectable gene transfer vectors is important for a number of potential cardiovascular gene therapies. This work provides, for the first time, proof of concept for this possibility through isolation and characterization of venous EC-selective peptides and AAV vectors. First, we demonstrate reduced virion accumulation in the major organs in which AAVwt is detected, predominantly the liver, for both modified AAVs, which translated to a significant reduction in hepatic transgene expression. Second, peptide-engineered AAVs had prolonged blood-circulating times compared with AAVwt, presumably because of the reduction in liver sequestration. Third, we observed enhanced gene delivery for AAVmtp into a specific vascular site in vivo at the level of virion accumulation and transgene expression. Retargeting was evaluated by real-time PCR to track virions and transgene expression by analysis of β-galactosidase to assess the ultimate aim, retargeting of transgene expression in vivo. Thus, these findings represent the first description of significantly altered AAV biodistribution in vivo after systemic administration. Further analysis of the selectivity of the retargeting is interesting. At
1 hour, both peptide-modified AAVs and AAVwt could be detected in the vena cava, and neither AAVmsl nor AAVmtp required heparin-dependent mechanisms. Importantly, at 28 days, with a low dose of each AAV, only AAVmsl and AAVmtp could be detected in the vena cava, providing evidence that in vitro venous selectivity was borne out in vivo. This finding for AAVwt is supported by the work of Seisenberger et al, who found that cell-surface docking can take place without cell infection as virions escape into the extracellular milieu. The in vitro transduction data also suggest that AAVwt-mediated transduction into hepatocytes is more dependent on HSPG binding than for ECs. It was shown previously that ECs express high levels of HSPG; however, they are present in the extracellular matrix, sequestering AAV away from the cell surface, preventing infection. Because AAVmtp produced residual heparin-dependent transduction in hepatocytes, incubation of ECs with heparin blocks its sequestration into HSPG in the extracellular matrix, enabling transduction via interaction of the peptide with the cell surface.

AAV-2 has a predominantly hepatic tropism when delivered systemically. Although primary and coreceptors have been identified for AAV-2, the mechanisms that dictate tropism after systemic administration are not entirely elucidated. We previously reported that insertion of a small targeting peptide at residue 587 conferred heparin-independent transduction and significantly reduced AAV-2 infection of nontarget cells. This dual phenotype is hypothesized to be a result of the close physical association between the peptide insertion and the HSPG binding motif. This raises the possibility of simultaneously detargeting and retargeting AAV-2 through single-step insertion of targeting peptides.

The tropism modification was generated at the level of the primary virus–cell interaction, an essential first step in the virion infection. The tropism of AAV for efficient transgene expression is also modulated through efficient internalization, trafficking, and nuclear gene conversion. AAV targeting may ultimately require refining with the use of ligands that mediate selective cell-surface binding and virus trafficking. Enhanced gene transfer for cancer gene therapy has also recently been demonstrated after direct intratumoral injection of AAV targeted with the RGD-4-C peptide, which targets α integrins. Therapeutic applications in the vasculature via systemic delivery are varied and will require a repertoire of targeting peptides unique for individual applications, such as has been demonstrated in vivo phage panning. Clearly, phage display will afford the selection of peptides that target to arterial endothelium and sites of disease in vivo based on heterogeneity of target receptor expression. Incorporation of such peptides is needed to realize the full potential of this technology. In summary, this study demonstrates the ability to rationally design and construct genetically engineered “designer” gene therapy vectors for selective delivery of genes to target vascular cells in vivo.

Figure 5. AAV organ distribution and vascular retargeting 28 days after infusion. A, AAVwt, AAVmsl, and AAVmtp virion quantification in liver, spleen, and lung 28 days after infusion of 1.5 × 10^10 GPs/animal (*P < 0.05 vs AAVwt, n = 4 mice/group). B, Real-time PCR quantification in liver 28 days after injection of 3 × 10^11 GPs/mouse (*P < 0.05 vs AAVwt, n = 4 mice/group). C, AAV quantification in vena cava 28 days after infusion of 1.5 × 10^10 (n = 4 mice/group; U.D., undetectable) or 3 × 10^11 GPs/mouse (*P < 0.05 vs AAVwt, n = 3 mice/group).

Figure 6. Transgene expression in vivo. A, Transgene expression in liver homogenates 28 days after infusion of 3 × 10^11 GPs/mouse of AAVwt or AAVmtp. *P < 0.05 vs AAVwt. B, Immunohistochemical detection of β-galactosidase expression in vena cava at 28 days after infusion. Representative sections from 4 animals/group. Arrows indicate examples of transgene-positive cells. Bar ~20 μm (applicable to all panels).
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References
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