Selective Targeting of Gene Transfer to Vascular Endothelial Cells by Use of Peptides Isolated by Phage Display

Stuart A. Nicklin, PhD; Steve J. White, PhD; Sarah J. Watkins, PhD; Robert E. Hawkins, MD; Andrew H. Baker, PhD

Background—Gene transfer to vascular cells is a highly inefficient and nonselective process, defined by the lack of specific cell-surface receptors for both nonviral and viral gene delivery vectors.

Methods and Results—We used filamentous phage display to isolate a panel of peptides that have the ability to bind selectively and efficiently to quiescent human umbilical vein endothelial cells (HUVECs) with reduced or negligible binding to nonendothelial cells, including vascular smooth muscle cells and hepatocytes. By direct biopanning on HUVECs and a second approach involving preclearing steps before panning on HUVECs, we isolated and sequenced 140 individual phages and identified 59 peptides. We selected 7 candidates for further investigation by secondary screening of homogeneous phages on a panel of cell types. Using adenovirus-mediated gene transfer as a model gene delivery system, we cloned the peptide SIGYPLP and the positive control peptide KKKKKKK upstream of the S11e single-chain Fv (“adenobody”) directed against the knob domain of the adenovirus to create fusion proteins. Adenovirus-mediated gene transfer via fiber-dependent infection was blocked with S11e, whereas inclusion of the KKKKKKK peptide retargeted gene transfer. The peptide SIGYPLP, however, retargeted gene delivery specifically to endothelial cells with a significantly enhanced efficiency over nontargeted adenovirus and without transduction of nontarget cells.

Conclusions—Our study demonstrates the feasibility of using small, novel peptides isolated via phage display to target gene delivery specifically and efficiently to HUVECs and highlights their use for retargeting both viral and nonviral gene transfer to vascular endothelial cells for future clinical applications. (Circulation. 2000;102:231-237.)

Key Words: bacteriophages ■ peptides ■ gene therapy ■ viruses ■ cells

The utility of gene delivery vectors for vascular gene therapy is limited by the nonselective nature in which the vectors, either nonviral or viral, interact with the cell surface, resulting in transduction of numerous cell types in addition to the target cell(s). This is further compounded by the fact that both endothelial and vascular smooth muscle cells (VSMCs) are relatively refractory to gene delivery vectors compared with permissive cell types. In the context of adenoviral vectors, this is due to the low level of the Coxackie-adenovirus receptor,1,2 which is required for efficient virus-cell interaction. There is therefore a requirement to generate gene delivery vehicles that have an increased affinity for vascular cells.

Vascular endothelial cells are an attractive target for many gene-therapeutic applications, including targeting endothelium in atherosclerosis, hypercholesterolemia, postangioplasty restenosis, hypertension, and transplantation. Furthermore, because of the proximity of the endothelium to the bloodstream, it is an attractive target for pathologies in which production of soluble proteins into the bloodstream would be a suitable gene therapy. However, uptake of viral and nonviral gene delivery vehicles by endothelium after intravenous or systemic delivery is low compared with other cells.3,4

Previous studies have established that gene delivery vectors can be targeted to individual cell types, thus generating cell-specific gene-therapeutic vehicles.5–10 Adenoviruses have been retargeted to endothelium with candidate ligands such as E-selectin,11 which targets activated endothelium in inflammatory situations, and fibroblast growth factor12,13; however, the fibroblast growth factor receptor is expressed on cells other than endothelium. The isolation of novel random peptides that mediate selective and efficient attachment and internalization of gene delivery vehicles into quiescent endothelial cells has not been reported.
Here, using phage display, we report the isolation of peptides that have the ability to attach selectively to human umbilical vein endothelial cells (HUVECs). Furthermore, using recombinant adenovirus as a model gene delivery system, we demonstrate that these peptides evoke endothelial cell–specific gene transfer.

Methods

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. Cell culture reagents were obtained from Gibco BRL, unless otherwise stated. Endothelial cell growth factor was from Sigma. The HeLa and HepG2 cell lines were from the European Collection of Animal Cell Cultures. The PhD Phage Display Peptide Library Kit was from New England Biolabs and displays random linear 7-mer peptides constrained at their C-terminus on the PIII coat protein. von Willebrand factor (clone F8/86) and smooth muscle cell α-actin (clone 1A4) antibodies were purchased from Dako.

Cell Culture

HUVECs were cultured in DMEM supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mMol/L L-glutamine, and 10% (vol/vol) FCS. HUVECs were isolated on the basis of a modified version of the protocol described by Jaffe et al.14 HUVECs were cultured in DMEM supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mMol/L L-glutamine, 20% (vol/vol) FCS, and 1% (vol/vol) endothelial cell growth factor and used between passages 2 and 5. HUVECs were identified by immunofluorescence for von Willebrand factor. VSMCs were obtained from medial explants of human saphenous vein obtained from patients undergoing coronary artery bypass surgery15 and cultured in DMEM (4500 mg/L glucose) supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 3% FCS, 2 mMol/L L-glutamine, and 10% (vol/vol) FCS. HUVECs were incubated at 4°C for 15 minutes. For HUVECs, 1×10⁹ pfu of phage were added to stock phage library was incubated with cells for 1 hour at 4°C. The resulting phages were preincubated or stock phage library was incubated with cells for 1 hour at 4°C. The resulting phages were preincubated with cells for 1 hour at 4°C. Cell culture reagents were obtained from Gibco BRL, unless otherwise stated. Endothelial cell growth factor was from Sigma. The HeLa and HepG2 cell lines were from the European Collection of Animal Cell Cultures. The PhD Phage Display Peptide Library Kit was from New England Biolabs and displays random linear 7-mer peptides constrained at their C-terminus on the PIII coat protein. von Willebrand factor (clone F8/86) and smooth muscle cell α-actin (clone 1A4) antibodies were purchased from Dako.

Phage Display

Phage libraries were amplified, purified, and titered according to the manufacturer’s protocols. HUVECs were plated into 6-well plates and cultured until 2 days after confluence, then 1×10⁹ pfu of input phage library was used to pan confluent cultures of HUVECs (passages 2 to 4) in triplicate. First, we used a direct screening approach and biopanned HUVECs for 4 rounds. Second, we bio-panned on HUVECs after biopanning for 1 hour on 2 successive cultures of VSMCs, HepG2 cells, and peripheral blood mononuclear cells (preclearing steps). Before biopanning, growth medium was changed to 1 mL DMEM containing 1% BSA, and the plates were incubated at 4°C for 15 minutes. For HUVECs, 1×10⁹ pfu of preclarified or stock phage library was incubated with cells for 1 hour at 4°C. Cells were washed 5 times in ice-cold PBS/1% BSA for 5 minutes per wash. Weakly associated phages were eluted in 1 mL 0.2 mMol/L glycine (pH 2.2) for 10 minutes on ice, followed by neutralization with 200 µL Tris-HCl (pH 8.0). High-affinity phages (tightly bound phages) were isolated by lysing the cells in 1 mL of 30 mMol/L Tris-HCl/1 mMol/L EDTA (pH 8.0) for 1 hour on ice. Cell debris was removed and the supernatant recovered. Phages were amplified and titered between each round to ensure that 10⁹ pfu of input phages was used at the start of each successive round. After completion of biopanning, Escherichia coli ER2537 was infected with the resulting phages and plated, and individual phages were picked, amplified, and sequenced. For further binding studies, high-titer homogeneous populations of each phage were prepared. Briefly, 1×10⁹ pfu of each phage were biopanned on HUVECs, VSMCs, and HepG2 cells for 30 minutes at 4°C. Unbound phages were removed by stringent washing, and the resulting cell-associated phages were titrated. The percentage recovery was calculated for each cell type with a peptideless phage to allow for background binding.

S11 “Adenobody” Cloning

Candidate peptides were cloned into the S11 adenobody retargeting system.16 We modified the S11 construct in 2 ways. First, we inserted an enterokinase site at the C-terminal NolI site immediately upstream of the 6-His and myc tags using oligonucleotides (sense oligonucleotide, 5'-GGCCGCAAGAGCGAGACACAA3'; antisense oligonucleotide, 5'-GGCCGCTTCTGGTCGCTC3') to create ps11e (Figure 1). We next inserted retargeting peptides at the 5’ end of the S11e sequence encoding the anti-fiber single-chain Fv using a unique Ncol cloning site (5'-ATGGGCG-3'). As a positive control, we inserted the sequence encoding the peptide KKKKKK using overlapping oligonucleotides (sense, 5'-CATGGCCCAAGAGAAGAGAGAAGAAGAGAGGACGACGGCCGACGTCC3'; anti-sense, 5'-CATGGGAGCTGGCGCCGCGCCCTTTTCTTCTTCTTTCTTTTGCGC3') to create ps11ep(K). The italicized sequences represent the peptide encoding DNA, and the boldface ones represent a triglycine stuffer sequence used to maintain frame and provide spatial distance from the single-chain Fv. The targeting peptide SIGYPPL was cloned in a similar manner but with the following oligonucleotides: sense, 5'-CATGGCGCTCGATGGGATACCCAATCGA-3'; antisense, 5'-GGCGGCGATGGGATACCCAATCGA-3'; antisense, 5'-CATGGGAGCTGGCGCCGCGCCCTTTTCTTCTTCTTTCTTTTGCGC3') to create the ps11e-SIGYPPL vector.

Recombinant Fusion Protein Production

Fusion proteins were prepared from periplasmic preparations of E coli JM109. Five hundred milliliters of 2×TY medium containing 100 µg/mL ampicillin and 0.1% glucose was inoculated with E coli JM109 pretransformed with either ps11e, ps11ep(K), or ps11e-SIGYPPL and incubated until O.D.₆₀₀ₐₙ=0.8. IPTG was added to 1 mmol/L, further incubated at 30°C for 4 hours, and centrifuged at 6000g. The pellet was resuspended in 20 mL PBS/1 mmol/L EDTA and centrifuged at 38 000g for 30 minutes at 4°C. The resulting protein was purified on Ni-NTA columns (Qiagen). Protein was quantified by use of the bicinchoninic acid assay (Pierce) and Western blotting using an anti-tetra-His antibody (Qiagen).

Adenoviral Constructs

The adenovirus RAdCMV expresses a non–nuclear-targeted LacZ gene from the cytomegalovirus (CMV) immediate early promoter.17 Recombinant adenoviruses were cums chloride–bound and titred by standard techniques.

Infection Protocols and Gene Transfer Assays

HUVECs, VSMCs, or HepG2 cells (5×10⁴) in triplicate were infected for 16 hours at 37°C with 100 pfu/cell of either RAdCMV alone or RAdCMV preincubated for 1 hour at room temperature with between 1 and 15 µg of S11e, S11ep(U), or S11eSIGYPPL. The medium was changed, and the cells were incubated for 48 hours in complete medium. For histochemical analysis, cells were washed twice in sterile PBS, fixed, and stained with X-Gal stain (100 mMol/L sodium phosphate, pH 7.3 (77 mMol/L Na₂HPO₄, 23 mMol/L NaH₂PO₄), 1.3 mMol/L MgCl₂, 3 mMol/L K₂Fe(CN)₆, 3 mMol/L K₄Fe(CN)₆, and X-Gal (1 mg/mL)) for 16 hours at 37°C. Cells were washed in PBS, and the nuclei were counterstained with  

Figure 1. Modification of S11 adenobody for peptide retargeting and generation of fusion proteins. Overlapping oligonucleotides were cloned 5’ to anti-knob single-chain Fv (VH and Vκ) to create spatial arrangement similar to that found in phage library (ie, peptides free at their N-terminus and constrained at their C-terminus). PeiB leader sequence is cleaved on entry of fusion protein into bacterial periplasm to leave free peptide and single-chain Fv fusion protein, which can be extracted and purified.
We designed 2 independent strategies to isolate endothelial cell-targeting phages. First, we exposed postconfluent cultures of HUVECs to the phage library and completed 4 rounds of panning to identify peptide sequences. To define that we were selectively enriching the library for endothelium-targeting phages, after each round of biopanning we titrated phages (Table 1). A significant increase in the titer of phages was observed (Table 1). After 4 rounds, we isolated and sequenced 60 individual phages (Table 2). Fifty-eight different peptide sequences were identified, 1 peptide (LTAELTP) appearing twice, and another phage contained no inserted peptide sequence (Table 2). A number of motifs were identified. For example, the peptides IVAQPR, QPRLHH, and TRSQPL all contain the sequence QP(P/R)L (Table 2). Other examples include 2 further peptides, INSNAPG and INSVSPH, which contain the motif INS at the N-terminus (Table 2).

We next modified biopanning to include preclearing on successive cultures of VSMCs, hepatocytes, and peripheral blood mononuclear cells before biopanning on HUVECs. A further 80 individual phages were sequenced (Table 2). With this strategy, only 3 different peptides were isolated. The peptides SIGYPLP, MSPPGPA, and LSNFHSS appeared 45, 33, and 2 times, respectively. In addition, the peptides SIGYPLP and LSNFHSS appeared by both screening approaches, whereas MSPPGPA from the direct screening approach differed by only 1 amino acid from MSPPGPA isolated in the preclearing strategy. For all peptides that appeared more than once, the DNA coding sequences were identical (data not shown).

Preselection of Peptides From Phage Display Identifies Candidates for Retargeting

After selection of putative endothelium-specific peptides, pure high-titer stocks of homogeneous phages were generated

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<th>TABLE 1. Recovery of Phages From HUVECs</th>
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After each round of panning on HUVECs, phages were titered. Only tightly bound phages were selected for further experiments.

$^*P<0.05$ vs titer recovered from the previous round of panning ($n=3$).

<table>
<thead>
<tr>
<th>TABLE 2. Peptide Sequences Obtained From Panning HUVECs</th>
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Peptide sequences and their frequencies obtained from phage display by direct biopanning on HUVECs and biopanning after preclearing (numbers in parentheses). Potential motifs are indicated with underlining.
for further characterization of their binding characteristics to HUVECs, VSMCs, and HepG2 cells (Figure 2). Recovery of phages from VSMCs varied between 0% for NTGPNRV and 24.1±27.8% for LTAELTP compared with the recovery from HUVECs, and all 7 peptides tested were lower in VSMCs than HUVECs. Diversity was observed in the recovery of phages from HepG2 cells, varying from 8.9±1.8% for NTGPNRV to 260.0±36.0% for LTAELTP compared with HUVECs (Figure 2). We therefore selected the peptide SIGYPLP for further investigation because of its consistent appearance in biopanning and its limited binding to nonendothelial cell types compared with HUVECs.

**Modification of the S11e Adenobody Single-Chain Fv Retargets Adenoviral Tropism Defined by 7-mer Peptides**

We first modified the S11e adenobody to allow cloning of short peptides to create fusion proteins (Figure 1). We designed the cloning so that the peptides were fused to the N-terminus of the single-chain Fv and were therefore constrained at the C-terminus (identical to the peptide presentation within the phage library). We next established that the S11e protein (without a retargeting peptide) could block fiber-dependent entry of RAdCMV into different cell types (Figure 3). Preincubation of RAdCMV with S11e produced a dose-dependent reduction in the level of β-galactosidase expression from HepG2 cells (Figure 3, P<0.05, n=3). Different results were obtained with VSMCs and HUVECs. In the absence of S11e, RAdCMV infection into both VSMCs and HUVECs was much lower than that observed for HepG2 cells (Figure 3). For VSMCs, there was no significant reduction in β-galactosidase production in the presence of increasing concentrations of S11e, presumably because of the low level of fiber-dependent entry of adenovirus into VSMCs (Figure 3). Infection into HUVECs, however, was significantly inhibited by increasing concentrations of S11e (Figure 3, P<0.05, n=3).

**Inclusion of a Polylysine Peptide at the N-Terminus of S11e Retargets Adenoviral Infection**

We next cloned overlapping oligonucleotides encoding the peptide sequence KKKKKKK into S11e to create S11ep(K) and purified the fusion protein. We observed that the S11ep(K) redefined adenoviral tropism, with an increase in  β-galactosidase expression from HepG2 cells (Figure 3, P<0.01 vs RAdCMV alone).
infection of both HepG2 cells and HUVECs compared with cells incubated with S11e alone (Figure 4). In fact, for HUVECs, the level of transduction achieved with S11ep(K) was above that observed with RAdCMV alone, demonstrating the inefficiency of adenovirus entry into HUVECs (Figure 4).

The Peptide SIGYPLP Retargets Gene Transfer Selectively to Endothelial Cells

After cloning of the SIGYPLP peptide into S11e to create S11eSIGYPLP, we analyzed the ability of this peptide, isolated from the phage library, to redirect gene transfer to HUVECs. S11eSIGYPLP induced a pattern of β-galactosidase expression in nonendothelial cells (VSMCs and HepG2 cells) identical to that of S11e alone. However, S11eSIGYPLP caused a significant increase in HUVEC cell transduction compared with the level achieved with S11e or RAdCMV alone (Figure 5, \( P < 0.01, n = 3 \)). Furthermore, the level of β-galactosidase expression increased from 0.29±0.2 μg β-galactosidase for infection with adenovirus alone to 4.52±0.11 μg β-galactosidase/mg protein for SIGYPLP-mediated infection, representing a 15.5-fold increase in transduction efficiency and resulting in transduction levels similar to those seen for normal nontargeted adenoviral infection into HepG2 hepatocytes (Figure 5, \( P < 0.05, n = 3 \)).

Discussion

Because of the inability of current gene delivery systems to efficiently transduce endothelial cells compared with other cell types, we sought to identify small, novel peptide sequences that can be incorporated into gene transfer vectors to mediate selective and efficient vehicle-to-endothelial cell interactions. For this purpose, we used random-phage-display libraries expressing 7-mer peptides on their coat proteins to pan primary, postconfluent cultures of endothelial cells. We identified 59 peptides by 2 independent panning strategies and further characterized the binding of 7 peptides to HUVECs, VSMCs, and HepG2 hepatocytes. The peptide SIGYPLP, which was isolated by both panning procedures, demonstrated high-level binding to HUVECs but low-level affinity for both VSMCs and hepatocytes and was therefore cloned into the S11e adenoviral system. We discovered that inclusion of SIGYPLP not only targeted endothelial cells in a cell-specific manner but also increased transgene expression significantly higher than that achieved with nontargeted adenovirus.

Phage display has previously been demonstrated to be a feasible strategy for obtaining small peptides that facilitate protein interactions.18–22 We included a preclearing step to increase the efficiency of the biopanning by removal of peptides that could mediate interactions with ubiquitously expressed cellular receptors. Indeed, preclearing enriched for candidate peptides, because the phage containing peptide SIGYPLP appeared only once without preclearing but 45 times with preclearing. The success of the technique was highlighted when high-titer stocks of pure populations of these peptide-expressing phages were used in repeated biopanning experiments on HUVECs, HepG2 cells, and VSMCs. Here, all 7 peptides tested showed significantly higher recovery from HUVECs than VSMCs. This is similar to the results of Rajotte and Ruoslahti,23 who recovered 4-fold more phages that expressed the peptide CGFECVRQCPERC from cells positive for the target receptor, membrane dipeptidase, than the recovery for control phage without an insert.

The wide tropism of adenovirus is a disadvantage in many clinically applicable situations for gene therapy when tissue-restricted gene expression is required. Thus, the development of suitable retargeting systems for adenovirus has been the goal of many researchers. Systems developed for retargeting adenoviral vectors have included nongenetic modifications of the adenoviral fiber by use of Fab fragments of antibodies.
chemically conjugated to retargeting ligands, with primary specificity to a component of the adenoviral fiber and a secondary specificity for a cell-associated protein, or scFv fragments genetically fused to retargeting ligands. In our system, we observed a large enhancement of infection using SIGYPLP. This observation can only improve the safety profile for gene transfer when used to target gene expression from endothelial cells.

It has previously been demonstrated that small peptide motifs are able to mediate specific cellular interactions, e.g., integrins with RGD motifs. Previous studies that used the method of in vivo phage display have also demonstrated that phages expressing small peptides are able to home to specific vascular beds and act as drug delivery agents to specific cell types. However, although candidate integrin-targeting strategies using RGD motifs have been demonstrated to enhance adenoviral infection in general, a novel linear peptide obtained by panning a random library has not.

Although we used adenoviruses as a model gene transfer system in this study, the peptides we isolated may also be suitable for retargeting gene transfer by use of nonviral vectors such as liposomes or other viral systems such as adeno-associated viruses. In summary, novel peptides isolated from random phage display libraries can efficiently and effectively retarget gene transfer to cells normally relatively resistant to transduction, such as HUVECs. Our study highlights the potential applications of small peptides in clinical gene therapy protocols in which selective and efficient transduction of endothelial cells would be advantageous.

Acknowledgment

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