Use of a Phage Display Library to Identify Oligopeptides Binding to the Lumenal Surface of Polarized Endothelium by Ex Vivo Perfusion of Human Umbilical Veins

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Human endothelial-specific targeting peptides were identified by biopanning within freshly-obtained human umbilical cords. Umbilical veins were cleaned in situ and M13 phage display libraries were passed through the cords. Tightly bound phage were recovered following isolation of endothelial cells by collagenase digestion and homogenisation, allowing production of enriched phage libraries for subsequent rounds of panning. After five rounds of biopanning, five promising sequences were selected and the binding of the corresponding phage clones was compared in perfused umbilical veins. Each of these peptides showed substantial binding, although the clone encoding the heptapeptide KPSGLTY showed the greatest, some 89-times greater than insertless phage. Binding of this phage clone was examined to cells in vitro, where it demonstrated at least five-times greater binding to isolated human umbilical vein endothelial cells than to 911, SKOV3, B16F10 and Cos7 cells. These initial peptides may prove useful targeting agents for endothelial-selective delivery, and this powerful approach should be readily applicable to biopanning in a broad range of human vessels ex vivo.

Keywords: Phage display; Oligopeptide; Polarized vascular endothelium; Umbilical Veins

INTRODUCTION

The vascular endothelial cell forms the main interface between blood and tissues and constitutes an important effector site for many forms of intravenous therapy. Tissue-associated endothelial cells provide an important target for tissue-specific delivery of drugs and genes and they may constitute the only realistic target when extravasation of the therapeutic vector is limited by capillary permeability. Identification of ligands capable of mediating selective binding of therapeutic vectors to specific types of endothelium could provide useful means to enable selective intravenous targeting to specific tissues and organs.

Identification of peptide ligands using bacteriophage peptide display libraries (Barry et al., 1996) has recently shown great promise, allowing selection and purification of phage bearing active ligands for production of enriched libraries for repeated application. In this way, consensus peptide sequences are gradually iterated and effective ligands can be identified. This approach has yielded promising results in animals, where oligopeptides capable of homing to specific organs have been identified following repeated biopanning (Pasqualini and Ruoslahti, 1996; Arap et al., 1998; Rajotte et al., 1998; Houston et al., 2001; Odermatt et al., 2001). The use of the approach in humans is more limited, however, since recovery of target organs following intravenous phage administration is possible only in special circumstances (Arap et al., 2002b). In addition, the feasibility of repeated iteration, involving administration of enriched libraries isolated from one person into another, is likely to raise safety concerns over the possibility of transferring unknown infectious agents. Because of the restrictions on in vitro biopanning in human subjects, phage selection experiments against human endothelial targets have conventionally been performed using cells in vitro (Nicklin et al., 2000; White et al., 2001). De-differentiation of cells in vitro, however, is a well...
characterized phenomenon (Augustin et al., 1994; Zangani et al., 1999), limiting therapeutic relevance of such systems. Most importantly, in vitro culture systems usually cannot reproduce the polarized phenotype of cells in vivo. In the case of endothelial cells, this means that ligands may be identified against receptors that are not accessible from the appropriate compartment (e.g. the bloodstream) in vivo. Targeting systems that work well in vitro but fail through polarization and inaccessibility of receptors in vivo are well known in gene therapy (Walters et al., 1999). In order to address these problems we have been developing biopanning strategies that make use of intact human organs, perfused ex vivo immediately following resection to minimize loss of cellular phenotype and allowing design of anatomically relevant biopanning procedures.

In this first report, we have developed an ex vivo system to perform biopanning with a phage library on polarized human umbilical vein endothelial (HUVE) cells. Umbilical cords have been obtained immediately after normal delivery and maintained ex vivo by perfusion with nutrient medium at 37°C. Phage libraries have been introduced into the umbilical vein and peptide ligands identified that mediate phage binding to the luminal surface of polarized HUVE cells.

MATERIALS AND METHODS

Source of Umbilical Cords and Ex Vivo Perfusion Technique

Umbilical cords were donated by healthy volunteer women and obtained immediately after delivery at Birmingham Women’s Hospital. Approval for this study was obtained prior to experimentation from the South Birmingham Local Research Ethics Committee and all donors signed informed consent forms. In a typical phage biopanning experiment, two or three freshly-isolated cords were used to generate three 15-cm cord segments that were harvested by injection of 6 ml of collagenase (DMEM, Gibco-BRL Life Technologies Ltd.) supplemented with 10% FCS, 2 mM l-glutamine and 1% penicillin/streptomycin (Sigma Chemical Co., Dorset, UK), at 37°C, 5% CO₂.

Iteration of Peptides Binding the Luminal Surface of Polarized Umbilical Vein Endothelial Cells

The Ph.D-7 M13 heptapeptide phage display library (New England BioLabs Inc., Beverly, MA, USA) was used for biopanning. 2 × 10¹¹ plaque forming units (pfu) of phage library, diluted in 5 ml PBS, were injected into the umbilical vein through the catheter. After closure of the catheters using forceps, the cord segment, containing the phage library, was incubated for 1 h at 37°C. The PBS containing unbound phage was removed and discarded. The insides of the vein were washed for 30 min by perfusion with warm PBS (500 ml/cord segment) using a peristaltic pump (205U, Watson-Marlow Ltd., Cornwall, UK) continuously at 60 rpm. Finally, endothelial cells were harvested by injection of 6 ml of collagenase (1 mg/ml in PBS, Gibco-BRL Life Technologies Ltd.) into the umbilical vein via the catheter, followed by incubation at 37°C for 30 min before recovery of the suspension. The harvested cells were then centrifuged at 1500 rpm for 5 min to remove weakly binding phage, the cell pellet resuspended in PBS (1 ml), and strongly binding or internalised phage were rescued by homogenisation of the cells. The number of phage recovered was determined by titering on agar plates containing Escherichia coli strain ER2537 and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal, Bioline Ltd., London, UK), isopropyl-b thiogalactopyranoside (IPTG, Bioline Ltd., UK), and 1% penicillin/streptomycin (Sigma Chemical Co., Dorset, UK). Recovered phage were amplified by addition to an early log phase culture of ER2537 and grown for 5 h with vigorous shaking (200 rpm, 37°C). The resulting enriched phage library was purified from the culture medium by precipitation with poly(ethylene glycol), according to the manufacturer’s recommended protocol, concentrated, titered and used for subsequent rounds of biopanning. Triplicate experiments were performed throughout, each involving five rounds of biopanning on separate cord segments. Phage from the triplicates were never mixed, hence the triplicate iterations of peptide sequences are completely independent.

Isolation and Sequencing of Phage DNA

Individual phage clones were isolated, at random, from each triplicate experiment, from each of the five rounds of biopanning, and DNA was isolated from each according to
the sequencing kit manufacturer’s recommended protocol (Biosystems, Perkin Elmer, Foster, CA, USA). The resulting DNA was used for sequencing using the −96 primer together with the BigDye Terminator Cycle Sequencing Kit (Biosystems, Perkin Elmer). DNA sequences were determined using an automated ABI PRISM 3700 sequence detector (Perkin Elmer).

**Binding Study of Individual Phage Clones, and Mixtures of Clones, in Umbilical Veins**

After five rounds of biopanning, five phage clones were identified as showing substantial binding to umbilical veins. Binding activity of individual phage clones was determined by incubating $10^{10}$ pfu of each clone in individual cord segments (1 h, 37°C), with subsequent washing and titering of phage recovered as described above. The binding of a phage clone containing no oligopeptide insert (insertless) was used as a negative control. In order to compare the binding activity of the endothelial-binding clones, equal numbers ($2 \times 10^5$ pfu) of each clone were mixed together, the mixture was introduced into the umbilical vein (1 h, 37°C) and tightly bound phage were titered by plaque analysis. Sixty plaques were picked at random, sequenced, and the proportion (%) of each clone compared.

**Measurement of Binding of Phage Clones to Cells and Cell Lines**

The binding of selected phage clones was determined on HUVE cells (passage 2 and passage 0, the latter being used immediately after their isolation from the cord) and on confluent cultures of 911, SKOV3, B16F10 and Cos7 cells in 6 well plates. Cells were acclimatised to 4°C for 30 min, then washed briefly in PBS prior to addition to each well of 5 × $10^7$ pfu of the appropriate phage clone, diluted into 1 ml M199 (HUVE cells) or DMEM (other cells) containing 1% BSA. Phage were allowed to bind to cells (1 h, 4°C, with gentle agitation), supernatant media discarded, and the cells were washed four times for 5 min with cold PBS (including 1% BSA). Cell-associated phage were recovered by lysing the cells (30 mM Tris–HCl, 10 mM EDTA, pH 8.0, 1 ml/well) on ice for 1 h. The number of phage recovered was determined by titering multiple dilutions of eluted phage as described earlier.

**Immunohistochemical Study of Phage Binding to Umbilical Vein In Situ**

Selected phage clones ($1 \times 10^{12}$ pfu in 3 ml PBS including 1% BSA) were incubated (1 h, 37°C) in segments of fresh umbilical cord, as described above, with insertless phage used as a negative control. Medium was removed and cells were rinsed, as described above, then 5 mm lengths of cord were cut with a scalpel, fixed in 10% phosphate-buffered formalin (24 h, 4°C), and embedded in paraffin. Tissue sections were prepared and stained with anti-M13 monoclonal antibody (Amersham Pharmacia, Piscataway, NJ, USA, 1:10 dilution, 1 h) then with StreptABComplex Duet HRP mouse/rabbit antibody (DAKO, Ely, UK, 1:200 dilution, 30 min) at room temperature. Signal was revealed by the addition of 3,3′-diaminobenzidine tetrahydrochloride (DAB, DAKO).

**RESULTS**

**Iteration of Consensus Oligopeptide Sequences**

**Binding to the Umbilical Vein**

Five rounds of biopanning were performed, in triplicate, on intact umbilical veins using an oligopeptide phage display library. The total number of phage recovered from the cords increased eight-fold during the five rounds of panning.

Several individual phage clones were picked, at random, after each round of biopanning, their insert DNA was sequenced, and encoded peptides deduced. In replicate experiment 1 the sequence LSTPPLL appeared in two of the five clones sequenced in round 2. This sequence was also detected in rounds 3 and 4, although it was not found in round 5 (Fig. 1). The sequence KPSGLTY appeared in the third and fourth rounds, although it also disappeared in the fifth. In the fifth round, the sequence KVLPFYD was dominant, appearing in three of the eight clones sequenced. Replicate experiment 2 was dominated throughout by the sequence HAIYPRH, which accounted for four of the eight clones sequenced in round 5. The sequence LSTPPLL identified in replicate 1, also appeared in round 3 of replicate 2. Replicate experiment 3 yielded the sequence ASYTQPA (rounds 3 and 5), which did not appear in any other replicate, KQTLPSA that appeared in rounds 4 and 5 of replicate 2, and QAISRNA that appeared in round 4 of replicate 1. The dominant sequence in round 5 of replicate 3 was KPSGLTY (4 of the 10 clones sequenced), which had also been found in rounds 3 and 4 of replicate 1.

**Comparison of the Binding of Selected Phage Clones to the Umbilical Vein and to Isolated HUVE Cells**

The sequences HAIYPRH, KPSGLTY, KVLPFYD, LPITPLP and LSTPPLL were selected for further study as umbilical vein-binding oligopeptides. The abilities of corresponding phage clones to bind to umbilical veins were compared by incubating each clone individually within fresh cord segments, with determination of the numbers of tightly bound phage in each case. Binding was compared with that of insertless phage, incubated with segments of the same cord in each case. The phage clone encoding KPSGLTY showed the greatest binding, with 89 times more phage recovered compared with insertless phage (Fig. 2A). Similarly, phage clones encoding the peptides KVLPFYD,
LPLTPL, HAIYPRH and LSTPPLL showed 45, 22, 21 and 9 times more recovery than insertless phage, respectively.

To assess comparative binding of the five clones, equal numbers were mixed, introduced into umbilical vein segments and their binding assessed as described above. Tightly bound phage were recovered and 60 plaques were picked at random and sequenced. The KPSGLTY phage clone was dominant, accounting for 43% of all the phage recovered, while other clones each accounted for between 10 and 17% of the total (Fig. 2B).

Binding of the phage clones to isolated (passage 2) HUVE cells was also compared, to verify their ability to bind to endothelial cells. Phage clones were incubated separately with confluent cells at 4°C for 1 h before tightly bound phage were collected and titered. Again the KPSGLTY phage showed the greatest recovery, reaching 22 times more than insertless phage (Fig. 3A). The other phage clones showed between three and seven times more recovery than insertless phage.

On the basis of all these results, the oligopeptide KPSGLTY was selected as the most promising sequence for binding to the luminal surface of polarised umbilical vein endothelial cells.

**Assessment of Endothelial Selectivity of KPSGLTY Binding**

Binding of a phage clone expressing the KPSGLTY oligopeptide was evaluated on confluent cultures of 911, SKOV3, B16F10 and Cos7 cells, and compared with HUVE cells (passages 0 & 2). The greatest numbers of KPSGLTY phage were recovered from HUVE cells (over \(10^4\)well) and there was no obvious difference in recovery from quiescent (P0) and proliferating (P2) HUVE cells. In contrast, the numbers of phage recovered from non-endothelial cells were much lower, invariably <20% of that from HUVE cells, indicating a possible endothelial selectivity for the KPSGLTY peptide.

**Immunohistochemical Study of Binding of Phage Clones to Umbilical Vein In Situ**

Immunohistochemistry was used to characterise the binding of phage clones expressing KPSGLTY peptide to umbilical vein in situ. Phage clones were incubated in intact segments of umbilical cord before sectioning and antibody-based visualisation of phage binding. The KPSGLTY phage showed a strong binding at
the endothelium of the umbilical vein (Fig. 4), while, in contrast, the insertless phage gave no discernible signal. This verifies the ability of KPSGLTY to mediate attachment of phage to the lumenal surface of polarised human umbilical vein endothelium \textit{in situ}.

**DISCUSSION**

To overcome the ethical and legislative hurdles associated with biopanning in terminal patients we have developed an \textit{in vitro} panning approach using intact vasculature. Here we have examined the ability of phage display libraries to iterate oligopeptides capable of binding to the luminal surface of human umbilical veins. The peptide-presenting phage library used is based on a combinatorial library of random peptide heptamers fused to a minor coat protein (pIII) of M13 phage and contains $2.8 \times 10^9$ different sequences. One of the peptides selected, KPSGLTY, shows considerable promise in both level and endothelial selectivity of adhesion. Immunohistochemical detection in \textit{ situ} showed that the peptide binds to the lumenal endothelial surface, as expected, and \textit{ex vivo} the peptide mediates similar interactions with quiescent (P0) and proliferating (P2) endothelial cells, indicating that it does not bind a cell cycle-dependent receptor.

A feature of the biopanning procedure described here was the stochastic evolution of whole heptapeptides, rather than iteration of partial sequences. Our previous studies, using ligands to displace phage from receptors, have led to gradual evolution of oligopeptide structures. Analysis of the DNA sequence of clones expressing identical oligopeptides showed the same codon usage. Although individual amino acids in this library are usually encoded by one main codon (reflected in the same codon usage being found in clones expressing the same oligopeptide in completely different experimental replicates), this is likely to reflect the very high stringency
conditions employed that caused purification of relatively small numbers of phage after each round of selection. It was particularly interesting that some identical sequences were identified in completely separate replicate experiments.

The patterns of oligopeptides defined suggested considerable variation in receptors present in different cords. Because of the sequential nature of the biopanning experiment, whole sequences could be lost by panning in a cord that did not express the appropriate receptor. This may represent a problem for defining the best ligand for a specific patient, but means that sequences ultimately identified by sequential panning in different cords are likely to express receptors in a large percentage of individuals.

Several ligands binding endothelial cells have been identified, including VEGF, angiopoietins, FGF2 and some peptides identified by biopanning using phage display libraries on animals or in vitro (Folkman and Shing, 1992; Maisonpierre et al., 1997; Neufeld et al., 1999; Binetruy-Tournaire et al., 2000; White et al., 2001). The KPSGLTY sequence identified here was not completely homologous to sequences of any known proteins, and showed no significant homology with known endothelial binding ligands such as VEGF, angiopoietin, FGF2 (FASTA3 database, European Bioinformatics Institute, Cambridge, UK and National Center for Biotechnology Information database, Bethesda, MD, USA) or endothelium-binding peptides (Binetruy-Tournaire et al., 2000; White et al., 2001).

Some peptides antagonising growth factors are known to have no homology to them (Ballinger et al., 1999; McConnell et al., 1999; Binetruy-Tournaire et al., 2000), probably reflecting the relative absence of structural constraints in the peptides, although the KPSGLTY oligopeptide may also be binding an unrelated endothelial component.

It is important to use relevant human tissues for biopanning to identify targeting ligands, as molecules expressed on murine tissue are quite different. For example, prostate-binding peptides identified in mouse (Arap et al., 2002b) are quite different from those identified for human (Arap et al., 2002a), despite the work being performed by the same group in both cases. Lambda phage has been used previously as an intravenous antibiotic (Slopek et al., 1987), however, the administration of a genetically-engineered phage library to human volunteers is likely to raise safety concerns, most notably with the desirability of reiterative panning by repeated administration of enriched libraries to successive volunteers. The ex vivo biopanning method described here may have considerable benefits for investigation of targeting ligands for human endothelium, although it is presently unclear how much homology will be found in targeting ligands selected against different types of vasculature. We are presently developing the same approach for the identification of ligands targeting human tumour-associated endothelium by biopanning via the tumour-feeding artery in freshly resected human cancers.

The peptides identified here could be applied for targeting treatments such as gene therapy, either using the peptides to deliver viral (Fisher et al., 2001) or synthetic (Fisher et al., 2000) vectors, or even using the phage themselves as the vectors (Larocca et al., 1998; Poul and Marks, 1999; Monaci et al., 2001). Finally, this sort of ex vivo perfusion model also provides several opportunities for basic scientific studies that involve manipulation of the endothelium in situ (e.g. induction of hypoxia, mechanical wounding) and identification, by positive and negative selection, of oligopeptides binding receptors that are differentially expressed on damaged, apoptotic or necrotic endothelial component.

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