Journal of Drug Targeting, 2003 Vol. 11 (1), pp. 53–59

Taylor & Francis
healthsciences

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

FUKUTO MARUTA[a,*], ALAN L. PARKER[a], KERRY D. FISHER[a], PAUL G. MURRAY[b], DAVID J. KERR[a,†] and LEONARD W. SEYMOUR[a,†,‡]

[a]*Cancer Research UK Institute for Cancer Studies, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK;* [b]*Department of Pathology, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*

(Received 24 May 2002)

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 vivo* 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

*Present address: First Department of Surgery, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan.
†Present address: Department of Clinical Pharmacology, Oxford University, Radcliffe Infirmary, Oxford OX2 6HE, UK.
‡Corresponding author.

ISSN 1061-186X print/ISSN 1029-2330 online
DOI: 10.1080/1061186031000086063

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 lumenal 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 then washed with Dulbecco's phosphate-buffered saline (PBS, Gibco-BRL Life Technologies Ltd., Paisley, UK). Catheters were inserted into the umbilical vein at both ends of each cord segment and cold PBS (100 ml) was injected into each umbilical vein via the catheters to remove residual blood and wash the inside of the vein.

Cell Lines and Primary Endothelial Cells

HUVE cells were harvested by collagenase perfusion of umbilical cords, obtained as described above, and cells were maintained in Medium199 (M199, Gibco-BRL Life Technologies Ltd.) containing 20% fetal calf serum (FCS) containing FGF2 (fibroblast growth factor 2; 10 ng/ml) in gelatinised flasks. The collagenase perfusion technique is fully described elsewhere (Seymour *et al.*, 1996).

The cell lines SKOV3 (human ovarian carcinoma) and Cos7 (monkey kidney epithelium) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). B16F10 murine melanoma cells were a kind gift from Professor Ernst Wagner (IMC, Vienna, Austria), and 911 human embryonic retinoblasts were obtained from Leiden University Medical Centre (Leiden, The Netherlands) (Fallaux *et al.*, 1996). Cells were maintained in Dulbecco's modified Eagle's medium (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% CO_2.

Iteration of Peptides Binding the Lumenal 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. 2×10^{11} 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.). 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 (2×10^9 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 (1×10^{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, LPLTPLP 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,

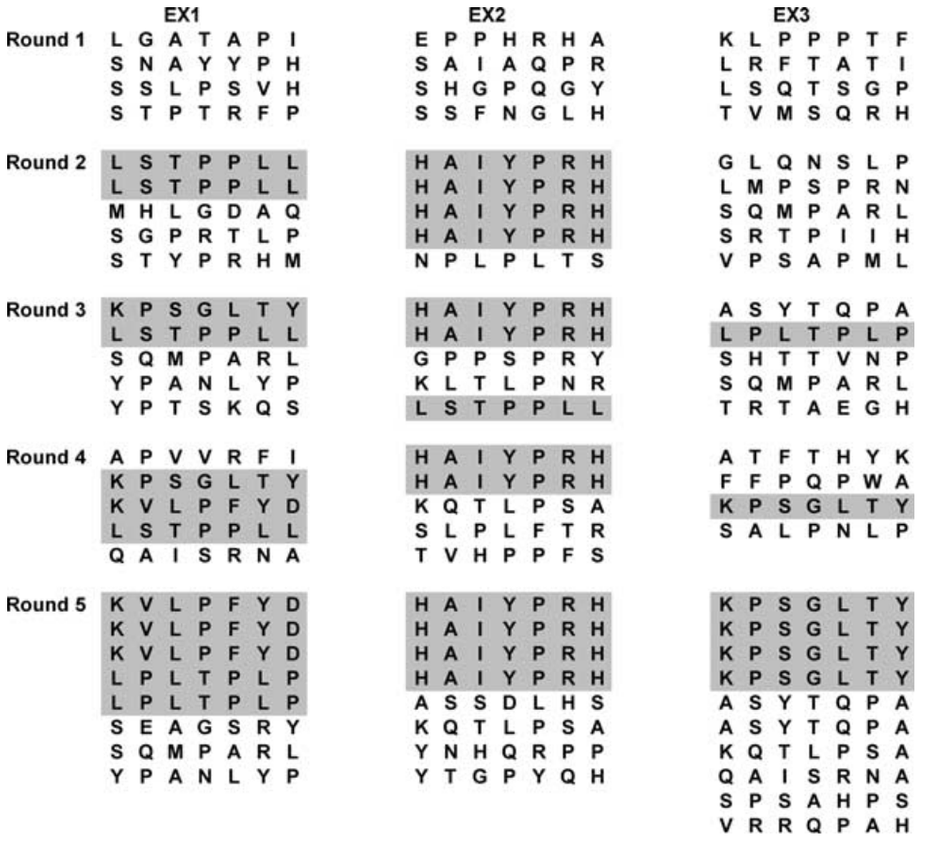

FIGURE 1 Identification of candidate peptides binding endothelial cells. DNA was isolated from individual plaques from each round of selection. The isolated DNA was sequenced across the insert region of pIII and the displayed peptide deduced. Binding peptides were iterated from each of five rounds of selection and from each of triplicate experiments run in parallel (Ex 1–3). The selected sequences, HAIYPRH, KPSGLTY, KVLPFYD, LPLTPLP and LSTPPLL are highlighted.

LPLTPLP, 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 lumenal 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

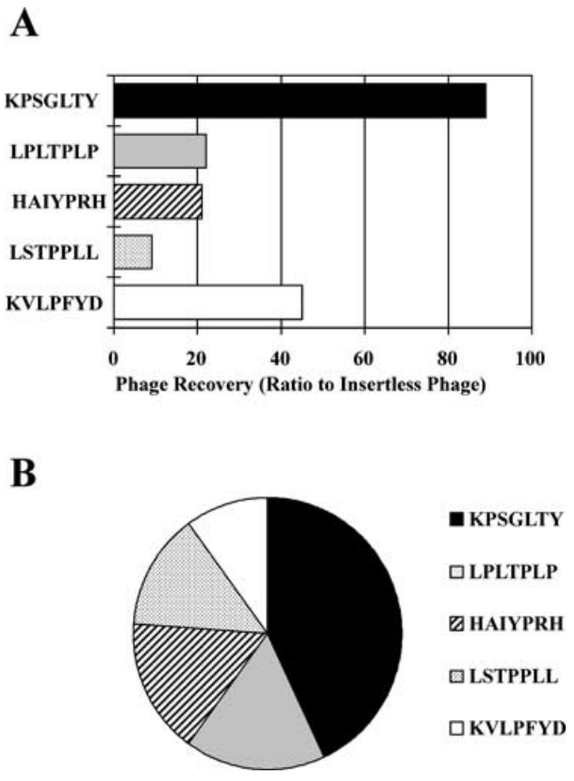

FIGURE 2 Selection of the best sequence binding to endothelial cells. HAIYPRH, KPSGLTY, KVLPFYD, LPLTPLP and LSTPPLL were selected as candidates for the best sequence binding to endothelial cells. (A) Binding studies on cords were performed individually using each of the five selected phage clones. To control for the size of the cord, the number of recovered targeted phage was assessed by comparison with that of insertless phage from the corresponding binding study performed simultaneously on the same cord. Numbers of phage recovered were as follows: KPSGLTY (3.82×10^6; insertless control 4.3×10^4), LPLTPLP (1.41×10^6; insertless control 6.48×10^4), HAIYPRH (1.15×10^6; insertless control 5.6×10^4), LSTPPLL (5.3×10^5;insertless control 6.0×10^4), KVLPFYD (1.19×10^6; insertless control 2.64×10^4). (B) Equal numbers of these five selected phage clones were mixed and incubated at 37°C inside an umbilical vein. Endothelial cells were removed by incubation with collagenase after washing inside the vein. Endothelial cell associated phage were collected and plaques of recovered phage were formed on X-gal, IPTG agar plates containing *E. coli* strain ER2537. Sixty plaques were picked at random and sequenced. They were found to represent KPSGLTY (25 plaques), LPLTPLP (10 plaques), HAIYPRH (9 plaques), LSTPPLL (8 plaques), KVLPFYD (6 plaques), unidentified (2 plaques) and the proportion (%) of each selected phage clone was calculated.

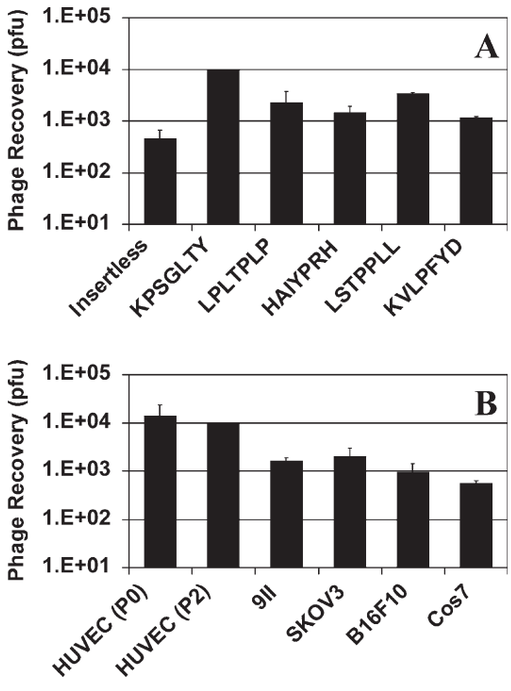

FIGURE 3 Phage binding assay on cell lines. To assess the affinity of selected phage clones for binding endothelial cells, 5×10^7 pfu of selected phage clones displaying the consensus motifs KPSGLTY, LPLTPLP, HAIYPRH, LSTPPLL, KVLPFYD or no peptide insert (insertless) were incubated on HUVE (passage 2) cells for 1 h at 4°C. Following extensive washing, bound phage were collected and quantified by plaque assay (A). Binding studies of KPSGLTY phage on HUVE (passage 0 and passage 2), 911, SKOV3, B16F10 and Cos7 cells were also performed (B).

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 *in situ*.

DISCUSSION

To overcome the ethical and legislative hurdles associated with biopanning in terminal patients we have developed an *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 lumenal 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 $\sim 2.8 \times 10^9$ different sequences. One of the peptides selected, KPSGLTY, shows considerable promise in both level and endothelial selectivity of adhesion. Immunohistochemical detection of binding *in situ* showed that the peptide binds to the lumenal endothelial surface, as expected, and *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

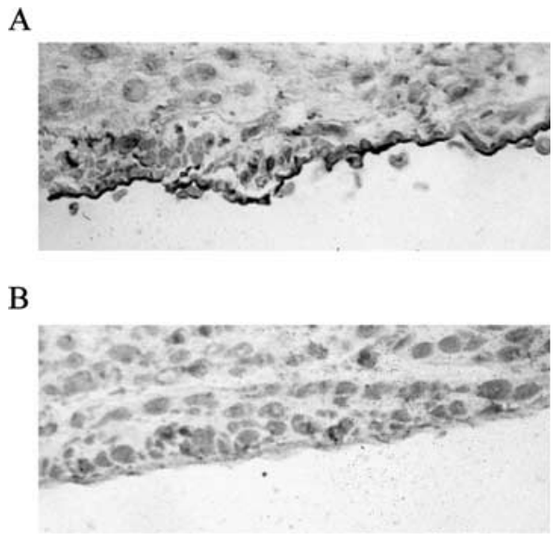

FIGURE 4 Immunohistochemical assessment of phage-binding on human umbilical vein *in situ*. Umbilical veins were incubated with 1×10^{12} pfu of either KPSGLTY phage or insertless phage for 1 h at 37°C. Following extensive washing of the inside of the vein, bound phage were detected *in situ* using an anti-M13 monoclonal antibody and StreptABComplex Duet HRP mouse/rabbit antibody. Signal was revealed by the addition of 3,3′-diaminobenzidine tetrahydrochloride. Significant signals were detected when cells were incubated with KPSGLTY-displaying phage (A) whilst no significant signal was detected when cells were incubated with insertless phage (B) (Magnification: × 400).

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 angiogenic vasculature, or on subendothelial surfaces that are exposed following endothelial destruction. These peptides may be particularly useful for targeting areas of vascular damage.

Acknowledgements

We are grateful to Shinshu University School of Medicine and Cancer Research UK for financial support. The technical assistance of the Functional Genomics Unit, School of Biosciences at Birmingham University is gratefully acknowledged. We thank Dr Richard Dales for critically reading and editing the manuscript.

References

Arap, W., Pasqualini, R. and Ruoslahti, E. (1998) "Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model", *Science* **279**, 377–380.

Arap, W., Haedicke, W., Bernasconi, M., Kain, R., Rajotte, D., Krajewski, S., Ellerby, H.M., Bredesen, D.E., Pasqualini, R. and Ruoslahti, E. (2002a) "Targeting the prostate for destruction through a vascular address", *Proc. Natl Acad. Sci. USA* **99**, 1527–1531.

Arap, W., Kolonin, M.G., Trepel, M., Lahdenranta, J., Cardo-Vila, M., Giordano, R.J., Mintz, P.J., Ardelt, P.U., Yao, V.J., Vidal, C.I., Chen, L., Flamm, A., Valtanen, H., Weavind, L.M., Hicks, M.E., Pollock, R.E., Botz, G.H., Bucana, C.D., Koivunen, E., Cahill, D., Troncoso, P., Baggerly, K.A., Pentz, R.D., Do, K.A., Logothetis, C.J. and Pasqualini, R. (2002b) "Steps toward mapping the human vasculature by phage display", *Nat. Med.* **8**, 121–127.

Augustin, H.G., Kozian, D.H. and Johnson, R.C. (1994) "Differentiation of endothelial cells: analysis of the constitutive and activated endothelial cell phenotypes", *Bioessays* **16**, 901–906.

Ballinger, M.D., Shyamala, V., Forrest, L.D., Deuter-Reinhard, M., Doyle, L.V., Wang, J.X., Panganiban-Lustan, L., Stratton, J.R., Apell, G., Winter, J.A., Doyle, M.V., Rosenberg, S. and Kavanaugh, W.M. (1999) "Semirational design of a potent, artificial agonist of fibroblast growth factor receptors", *Nat. Biotechnol.* **17**, 1199–1204.

Barry, M.A., Dower, W.J. and Johnston, S.A. (1996) "Toward cell-targeting gene therapy vectors: selection of cell-binding peptides from random peptide-presenting phage libraries", *Nat. Med.* **2**, 299–305.

Binetruy-Tournaire, R., Demangel, C., Malavaud, B., Vassy, R., Rouyre, S., Kraemer, M., Plouet, J., Derbin, C., Perret, G. and Mazie, J.C. (2000) "Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis", *EMBO J.* **19**, 1525–1533.

Fallaux, F.J., Kranenburg, O., Cramer, S.J., Houweling, A., Van Ormondt, H., Hoeben, R.C. and Van Der Eb, A.J. (1996) "Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors", *Hum. Gene Ther.* **7**, 215–222.

Fisher, K.D., Ulbrich, K., Subr, V., Ward, C.M., Mautner, V., Blakey, D. and Seymour, L.W. (2000) "A versatile system for receptor-mediated gene delivery permits increased entry of DNA into target cells, enhanced delivery to the nucleus and elevated rates of transgene expression", *Gene Ther.* **7**, 1337–1343.

Fisher, K.D., Stallwood, Y., Green, N.K., Ulbrich, K., Mautner, V. and Seymour, L.W. (2001) "Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies", *Gene Ther.* **8**, 341–348.

Folkman, J. and Shing, Y. (1992) "Angiogenesis", *J. Biol. Chem.* **267**, 10931–10934.

Houston, P., Goodman, J., Lewis, A., Campbell, C.J. and Braddock, M. (2001) "Homing markers for atherosclerosis: applications for drug delivery, gene delivery and vascular imaging", *FEBS Lett.* **492**, 73–77.

Larocca, D., Witte, A., Johnson, W., Pierce, G.F. and Baird, A. (1998) "Targeting bacteriophage to mammalian cell surface receptors for gene delivery", *Hum. Gene Ther.* **9**, 2393–2399.

Maisonpierre, P.C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S.J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T.H., Papadopoulos, N., Daly, T.J., Davis, S., Sato, T.N. and Yancopoulos, G.D. (1997) "Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis", *Science* **277**, 55–60.

McConnell, S.J., Thon, V.J. and Spinella, D.G. (1999) "Isolation of fibroblast growth factor receptor binding sequences using evolved phage display libraries", *Comb. Chem. High Throughput Screen.* **2**, 155–163.

Monaci, P., Urbanelli, L. and Fontana, L. (2001) "Phage as gene delivery vectors", *Curr. Opin. Mol. Ther.* **3**, 159–169.

Neufeld, G., Cohen, T., Gengrinovitch, S. and Poltorak, Z. (1999) "Vascular endothelial growth factor (VEGF) and its receptors", *FASEB J.* **13**, 9–22.

Nicklin, S.A., White, S.J., Watkins, S.J., Hawkins, R.E. and Baker, A.H. (2000) "Selective targeting of gene transfer to vascular endothelial cells by use of peptides isolated by phage display", *Circulation* **102**, 231–237.

Odermatt, A., Audige, A., Frick, C., Vogt, B., Frey, B.M., Frey, F.J. and Mazzucchelli, L. (2001) "Identification of receptor ligands by screening phage-display peptide libraries *ex vivo* on microdissected kidney tubules", *J. Am. Soc. Nephrol.* **12**, 308–316.

Pasqualini, R. and Ruoslahti, E. (1996) "Organ targeting *in vivo* using phage display peptide libraries", *Nature* **380**, 364–366.

Poul, M.A. and Marks, J.D. (1999) "Targeted gene delivery to mammalian cells by filamentous bacteriophage", *J. Mol. Biol.* **288**, 203–211.

Rajotte, D., Arap, W., Hagedorn, M., Koivunen, E., Pasqualini, R. and Ruoslahti, E. (1998) "Molecular heterogeneity of the vascular endothelium revealed by *in vivo* phage display", *J. Clin. Investig.* **102**, 430–437.

Seymour, L.W., Shoaibi, M.A., Martin, A., Ahmed, A., Elvin, P., Kerr, D.J. and Wakelam, M.J. (1996) "Vascular endothelial growth factor stimulates protein kinase C-dependent phospholipase D activity in endothelial cells", *Lab. Investig.* **75**, 427–437.

Slopek, S., Weber-Dabrowska, B., Dabrowski, M. and Kucharewicz-Krukowska, A. (1987) "Results of bacteriophage treatment of suppurative bacterial infections in the years 1981–1986", *Arch. Immunol. Ther. Exp.* **35**, 569–583.

Walters, R.W., Grunst, T., Bergelson, J.M., Finberg, R.W., Welsh, M.J. and Zabner, J. (1999) "Basolateral localization of fiber receptors limits adenovirus infection from the apical surface of airway epithelia", *J. Biol. Chem.* **274**, 10219–10226.

White, S.J., Nicklin, S.A., Sawamura, T. and Baker, A.H. (2001) "Identification of peptides that target the endothelial cell-specific LOX-1 receptor", *Hypertension* **37**, 449–455.

Zangani, D., Darcy, K.M., Masso-Welch, P.A., Bellamy, E.S., Desole, M.S. and Ip, M.M. (1999) "Multiple differentiation pathways of rat mammary stromal cells *in vitro*: acquisition of a fibroblast, adipocyte or endothelial phenotype is dependent on hormonal and extracellular matrix stimulation", *Differentiation* **64**, 91–101.