Combinatorial Selection of Cell-Specific Peptides by Phage Display Experiments

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ABSTRACT

Peptides that recognize specific cell types promise to be valuable tools both in research and clinical applications. We report the discovery of novel peptides that are highly specific for endothelial progenitor cells (EPC) by screening phage display libraries. Our strategy for isolation of ligand peptides is designed to allow using whole cells as an affinity matrix. We show that binding of phage clones is highly specific for EPC since no binding has been observed on human umbilical vein endothelial cells (HUVEC), HL-60 leukemia cells, human neutrophils and monocytes. To demonstrate the utility of the phage display selected, EPC-specific peptide sequences, free peptides have been synthesized and covalently immobilized to synthetic materials using chain transfer free radical polymerization chemistry. The results from this study contribute to the development of new strategies to be exploited for the design of biomimetic materials for tissue engineering applications.

Keywords: cell-specific peptides, phage display libraries, endothelial progenitor cells, biomimetic materials, tissue engineering

1 INTRODUCTION

The discovery of new peptide ligands that recognize specific cell types promise to be a valuable tool both in research and clinical applications. Cell-type specific peptides can be used as drug delivery vehicles, diagnostic agents, affinity reagents for cell purification, gene therapy delivery agents and research tools to probe the molecular diversity of a cell surface [1]. Screening libraries to identify cell-selective peptides using whole cells as an affinity matrix has many advantages: no prior information is required about the putative receptor(s) and selection can screen for cellular uptake as well as binding. Screening intact cells enables the identification of cellular receptors that may not have been considered as targets or have not yet been identified. Furthermore, this approach ensures that the selected peptide sequence binds to its target in the presence of many other biological macromolecules and allows for selection of membrane proteins that are often difficult to express and purify.

Endothelial progenitor cells (EPC) are bone-marrow derived cells that have the capacity to proliferate, migrate and differentiate into endothelial cells [2]. The first evidence indicating the presence of EPCs in the adult circulation emerged when a small subset of mononuclear blood cells was shown to acquire an endothelial cell-like phenotype *in-vitro* and to incorporate into capillaries *in-vivo* [3]. EPCs have been investigated with regard to their pro angiogenic/vasculogenic potential. It has been shown that EPC increase perfusion in murine models of hind limb ischemia [4]. Recently, EPC have been applied in the field of tissue engineering as a means of improving biocompatibility of vascular grafts. Artificial grafts, first seeded with autologous CD34+ cells from canine bone marrow and then implanted into the aorta were found to have increased surface endothelialization and vascularization compared to controls [5]. An important advantage of using EPC-derived cells for these applications is that the cells can be obtained from peripheral blood. This eliminates the need to sacrifice a blood vessel or tissue to obtain endothelial cells. Although the number of EPC in adult peripheral blood is low it has been shown that when isolated and expanded in culture EPC can undergo more than 1000 population doublings [6]. This doubling ability is in contrast to that of mature EC that can be grown in culture but exhibit senescence after about 30 population doublings. These findings make EPC an attractive cell source for tissue engineering applications.

In this investigation we performed non-biased selection using an outgrowth of endothelial progenitor whole cells as an affinity matrix. To avoid non specific binding we applied negative-positive selection approach by pre-incubating the library with non EPC. We show that binding of phage clones is highly specific for EPC since no binding has been observed on human umbilical vein endothelial cells (HUVEC), HL-60 leukemia cells, human neutrophils and monocytes. To demonstrate the utility of the phage display selected, EPC-specific peptide sequences, free peptides have been synthesized and covalently immobilized to synthetic surfaces to design biomimetic materials to be used as tissue engineering scaffolds.

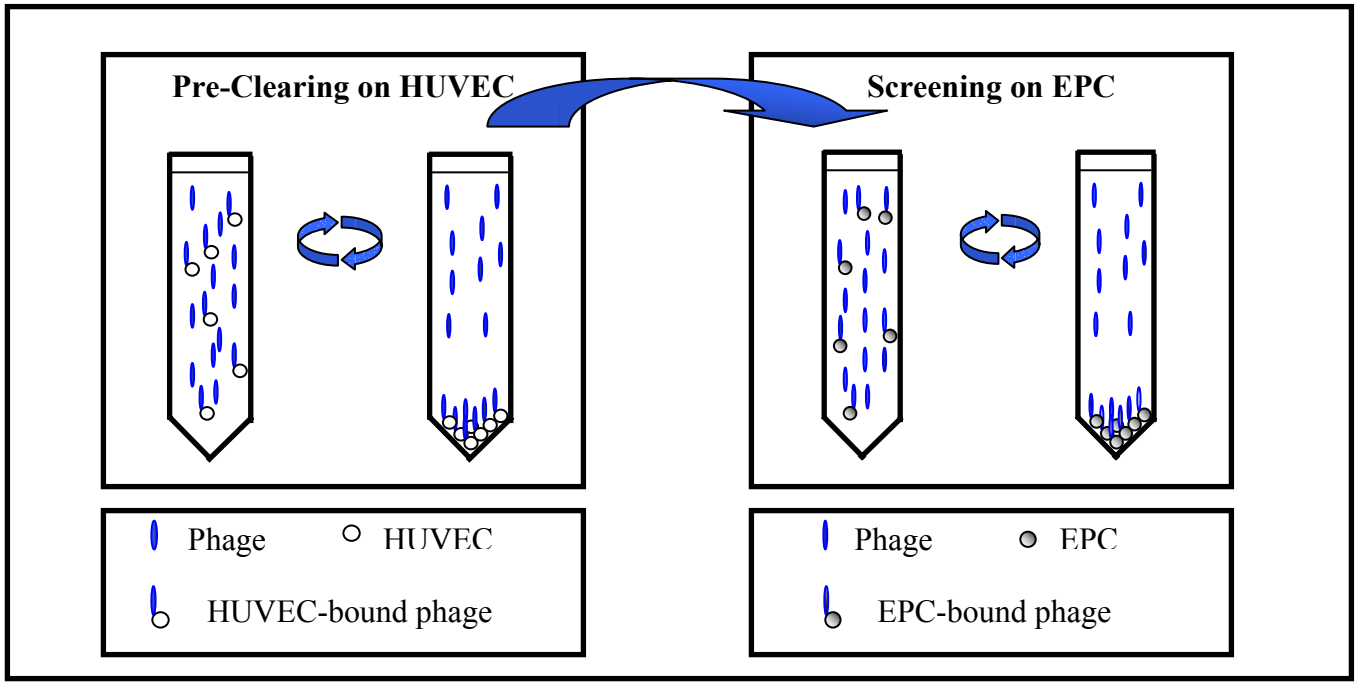

Figure 1: Panning strategy to isolate phage that bind specifically to endothelial progenitor cells. To avoid non-specific binding negative-positive selection approach was implemented. First, the peptide phage library was pre-cleared by incubating with HUVEC. After centrifugation the supernatant containing unbound phage was transferred to EPC cell suspension. After incubation, cells and bound phage were separated by centrifugation. Cell-bound phage in the pellet were rescued by infection, amplified and used for another round of biopanning.

2 EXPERIMENTAL

2.1 Cells and Culture Conditions

Endothelial culture from peripheral blood: Fresh blood was collected from healthy volunteer donors by venipuncture and anticoagulated with buffered sodium citrate. The anticoagulated blood was diluted 1:1 with HBSS (Sigma) containing 1 mM EDTA and 0.5% BSA. Buffy coat mononuclear cells were obtained from diluted blood by density centrifugation method using Histopaque 1077 (Sigma). The cells were washed in PBS three times at 400g for 10 min before culturing. Buffy coat mononuclear cells from 50-100 ml were re-suspended in EGM-2 medium (Clonetics, Inc., San Diego, CA) without further subpopulation enrichment procedures and placed into one well of a six well plate coated with type 1 collagen (BD Biosciences, Bedford, MA). The plate was incubated at 37^{o}C in a humidified environment with 5% CO_2. Culture medium was changed daily. Cells were passaged first at about 4 weeks with trypsin-EDTA on tissue culture plates coated with 50 μg/ml of fibronectin as substrate.

Isolation of human monocytes and neutrophils: Monocytes and neutrophils were obtained from the same preparation. Monocytes were collected from the supernatant of the cultured buffy coat mononuclear cells that were allowed to adhere overnight. The cell concentration was adjusted to 1 x 10^5 cells per ml.

Neutrophils were collected from the lower portion of the density gradient preparation. The upper layers were removed and processed as described above. The neutrophil rich Histopaque layer was transferred to a centrifuge tube and diluted in fresh RPMI to wash the cells free of Histopaque. The suspension was centrifuged at 700 x g for 15 min at room temperature. The supernatant was aspirated and the pellet resuspended in 10 ml of RPMI and centrifuged at 700 x g for 10 min at room temperature. Contaminating red cells were lysed by quickly resuspending the pellet in sterile water at 4^{o}C. After 30 s an equal volume of 3 mol l^{-1} saline solution (1.8%) was added in order to return the solution to isotonicity. The suspension was then centrifuged for 10 min at 250 x g at 4^{o}C. The cell lysis step was repeated. The final pellet was re-suspended and concentration adjusted to 1 x 10^5 cells per ml.

Human umbilical vein endothelial cells (HUVEC) were from the American Type Culture Collection (ATCC, Manassas, VA). Passages 4 to 8 were used in this study. HUVEC were cultured in EGM-2 medium (Clonetics) at 37^{o}C in an incubator with humid atmosphere and 5% CO_2.

Peripheral blood human HL-60 promyelocytic cells from the American Type Culture Collection (ATCC) were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum.

2.2 Bacteriophage Panning

Cells at 80% confluence were detached by treating with 0.25% trypsin-EDTA, washed once with EGM-2 medium and re-suspended in EGM-2 containing 1% BSA at 1 x 10^5 cells per ml. In the pre-clearing step, 1 x 10^5 HUVEC were incubated with 10 µl of PhD-12 peptide phage display system (New England Biolabs, Beverly, MA) within 1.5 ml Eppendorf tubes for 2 hours on ice; the mixture was then centrifuged. In a screening step, the unbound phage pool remaining in the supernatant was transferred to a fresh tube and incubated with 1 x 10^5 EPC. After 1 hour incubation on ice, the cell-phage complexes were separated by centrifugation. After three intensive washes with TBS-0.5% Tween-20 buffer the bound phage was non-specifically eluted with 0.2 M Glycine-HCl buffer (pH 2.2) for 10 min. The eluate was immediately neutralized by 1M Tris.HCl buffer (pH 9.0). An aliquot of the eluted phage was used for determining titer by plaque assay. The rest of the phage eluate was amplified in mid-log phase *E. coli* ER 2738, and purified by precipitation with polyethylene glycol. An aliquot of the amplified phage (1.5 x 10^{11} pfu) was subsequently re-applied to newly trypsinized cells for a total of three biopanning rounds and two amplification steps.

2.3 Assaying for Binding Specificity

The specificity of EPC-selected phage clones was determined by biopanning on a panel of other cell types. The biopanning procedure was carried as described above with the exception of including the pre-clearing incubation.

2.4 Biomaterial Synthesis and Peptide Incorporation

Peptide grafted materials were prepared by a chain transfer free radical polymerization reaction as described by Fussell and Cooper [7, 8]. The monomers used in the reaction were n-hexyl methacrylate (HMA) (Alfa Aesar, Ward Hill, MA), methyl methacrylate (MMA) (ACROS Organics, Pittsburgh, PA), and methacrylic acid (MAA) (ACROS Organics, Pittsburgh, PA). The polymerization reaction was performed in dimethyl formamide (DMF) (Sigma-Aldrich, Milwakee, WI) using 2, 2-azobisisobutyronitrile (AIBN) (Aldrich Chemical, Milwakee, WI) as the initiator. The molar ratios of the monomers in the feed were 90 mol% HMA, 8 mol% MMA, and 2 mol% MAA. The amount of the initiator comprised 0.01 mol% of the total monomer content. The reaction was carried out overnight at 55-60^{o}C [9].

The peptide sequence MPTLTRAPHTAC used in this research was synthesized at UNC Protein Synthesis Core Facility (UNC, Chapel Hill, NC). The cysteine residue at the C-terminus of the peptide allowed for chain transfer chemistry. The peptide was added to the monomer mixture after the solvent had been purged with argon and the reaction was carried overnight at 55-60^{o}C [7, 8].

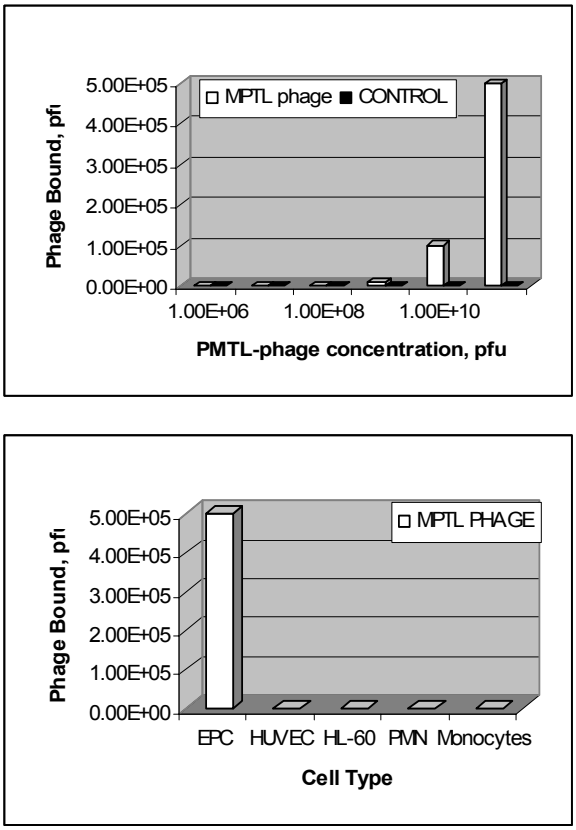

Figure 2: (Top) Cell-binding assay with MPTL-phage ligand. Increasing phage amounts were incubated with 1 x 10^5 EPC. The bound phage was determined by plaque titering assay (□).Control experiments were performed in the absence of EPC to exclude binding to the plastic of the microfuge tube (■). (Bottom) Cell specificity assay of EPC-selected MPTL-phage. Selectivity determinations were made for MPTL-phage and the cell lines shown. Significant binding of MPTL-phage was not observed with any other cell type tested.

3 RESULTS AND DISCUSSION

3. 1 Screening Endothelial Progenitor Cells

We designed two-step panning strategy to isolate phage that bind specifically to cultured endothelial progenitor cells. First, to decrease non-specific binding we pre-cleared the phage library on non EPC. We incubated the phage library with HUVEC and centrifuged to separate HUVEC-phage complexes and unbound phage clones. Second, the unbound phage pool was incubated with cultured EPC for 1 hour on ice. EPC-bound phage were separated by centrifugation, recovered by bacterial infection, amplified and subjected to two more rounds of selection (Figure 1).

3.2 Analysis of Phage Displayed Peptides

To test the selection method, after the third round of biopanning the eluate was titered, individual phage clones were isolated and amplified. The DNA of randomly chosen phage clones was sequenced and the encoded peptide sequences were deduced. Below we report results from *in-vitro* phage binding assays for a representative phage clone displaying the motif MP(P/T)L, termed MPTL-phage.

We evaluated the binding of MPTL-phage to cultured endothelial progenitor cells by incubating equal amounts of cells (1 x 10^5) with increasing amounts of phage ranging from 10^6 pfu to 10^{11} pfu. Control experiments were performed in parallel in the absence of EPC to exclude phage binding to the plastic walls of the container. As seen from Figure 2, MPTL-phage binds to EPC in a concentration dependent manner.

In order to test the specificity of MPTL-phage, equal amounts of phage (1 x 10^{11} pfu) were incubated with different cell lines including HUVEC, HL-60, human neutrophils and monocytes. MPTL-phage did not exhibit enhanced binding to any of the other cell types tested, as shown on Figure 2 (bottom panel). Of note is the fact that MPTL-phage was selected with negative screening procedure only against HUVEC.

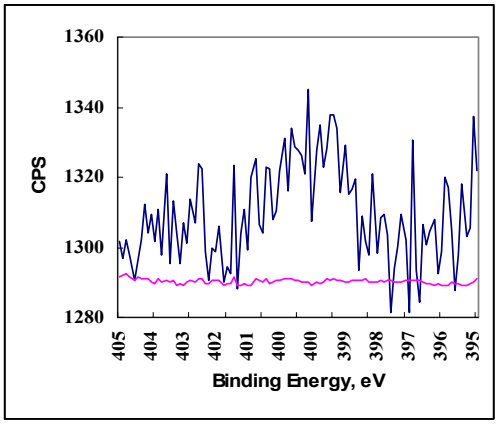

Figure 3: XPS spectra for N 1s of peptide grafted material and a control sample.

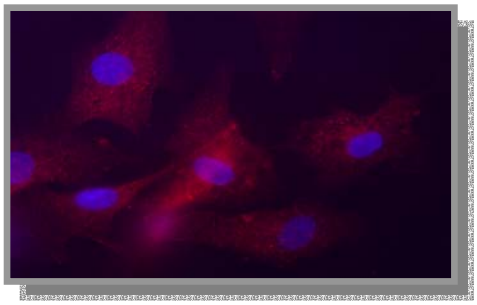

Figure 4: Adhesion and spreading of endothelial progenitor cells on peptide grafted surface after 4 h incubation at 37°C. Cells were labeled to visualize the actin cytoskeleton (in red); the nuclei were stained in blue.

3.3 Peptide Grafted Biomaterial Synthesis and Characterization

Methacrylate based terpolymers were synthesized with active peptide sequences through chain transfer reaction during polymerization. The peptide incorporation was determined by X-ray photoelectron spectroscopy (XPS) analysis. Figure 3 shows XPS spectra for N 1s peptide grafted material and a control sample.

To confirm that the biological activity of peptides identified by phage display screening is retained when they are covalently immobilized to synthetic surfaces, we studied cellular adhesion. Figure 4 demonstrates that these materials support cell attachment and spreading and thus merit further evaluation for use as tissue engineering scaffolds.

Acknowledgements: This work was partially supported by NIH grant R01 HL61656.

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