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Selective endothelial cell attachment to peptide-modified terpolymers

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ABSTRACT

In a previous report we screened a combinatorial peptide library to identify novel ligands that bind with high affinity and specificity to human blood outgrowth endothelial cells (HBOEC). In this study we demonstrate the use of the phage display-selected-HBOEC-specific peptides as a tool to direct and modulate endothelial cell (EC) behavior with a focus on designing functional biomaterials intended for use in cardiovascular applications. First, we ensured that our peptide ligands did not interfere with EC function as tested by proliferation, migration, tube formation, and response to vascular endothelial growth factor. Second, peptides that supported EC function were incorporated into methacrylic terpolymers via chain transfer free radical polymerization. The HBOEC-specific peptide, TPSLEQRTVYAK, when covalently coupled to a terpolymer matrix, retained binding affinity towards HBOEC in a serum-free medium. Under the same binding conditions, the attachment of human umbilical vein endothelial cells (HUVEC) was limited, thus establishing HBOEC specificity. To our knowledge, this is the first report demonstrating specificity in binding to peptide-modified biomaterials of mature EC, i.e., HUVEC, and EC of progenitor origin such as HBOEC. The findings from this work could facilitate the development of autologous cell therapies with which to treat cardiovascular disease.

1. Introduction

A common approach to direct cell and tissue responses at synthetic surfaces is to modify the material to mimic the extracellular matrix [1,2]. Typically, short recognition sequences presenting cell binding motifs found in components of the extracellular matrix (e.g., the RGD or the YIGSR peptides) are immobilized on the material to promote cell adhesion via ligand–receptor interactions [3–7]. Although surface functionalization remains one of the most promising strategies, the clinical utility of this approach is limited by the need to develop materials that are optimized to promote adhesion of a specific cell type. Towards this end, we designed and implemented a high-throughput protocol for screening a phage display peptide library to identify and select novel peptide ligands that bind with high affinity and specificity to human blood outgrowth endothelial cells (HBOEC) [8]. In this work we explore the ability of these HBOEC-specific peptides to modulate endothelial cell (EC) function when covalently attached to a synthetic material.

Blood outgrowth endothelial cells (BOEC) have recently been applied to the field of tissue engineering as a means of improving biocompatibility of vascular grafts. Artificial small diameter vascular grafts, seeded with BOEC derived from canine peripheral veins, were found to have increased surface endothelialization and vascularization compared to controls in a canine carotid artery model [9]. Similarly, when cultured autologous ovine endothelial progenitor cells were seeded into carotid interposition grafts, the seeded grafts achieved physiological motility and remained patent for 130 days vs. 15 days in nonseeded grafts [10]. An important advantage of BOEC for these applications is that the cells can be obtained from peripheral blood. Further, it has been shown that when isolated and expanded for 1 month, BOEC can undergo more than 1000 population doublings, which is in stark contrast to the approximate 20 population doublings of mature EC grown for the same period of time [11]. These findings justify the use of BOEC isolated from peripheral blood and expanded in vitro as a suitable source of autologous cells for constructing functional tissue engineered blood vessel replacements. Alternatively, endothelial progenitors recruited directly from blood could be used to promote endothelialization on a synthetic scaffold material. This facilitated endogenous repair is a novel approach to tissue engineering that avoids in vitro culture of autologous cells while minimizing the

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invasiveness of associated clinical procedures. This strategy relies on harnessing the intrinsic regenerative power of endothelial progenitor cells to initiate healing processes in situ [12,13].

In spite of the enormous potential of the BOEC population for clinical applications in treating cardiovascular disease, the molecular characteristics, especially with regard to the expression of cell surface markers, are poorly understood. To discover endothelial progenitor cell-specific ligands we performed non-biased selection from a phage display peptide library using HBOEC as an affinity matrix [8]. In this study we used the phage display-selected-HBOEC-specific peptide ligands as a tool to direct and modulate EC behavior with a focus on designing functional materials intended for use in cardiovascular applications. First, we ensured that our peptides did not interfere with EC function as tested by proliferation, migration, tube formation, and response to vascular endothelial growth factor (VEGF). Second, peptides that supported EC function were covalently immobilized to methacrylic terpolymers via chain transfer free radical polymerization and tested to see if they retain their binding activity when coupled to surfaces. Our results demonstrate that methacrylic materials modified with phage display-selected ligands elicit cell-specific responses.

2. Materials and methods

2.1. Isolation of human blood outgrowth endothelial cells from peripheral blood

The use of human material described in this study was approved by the responsible ethical committee. Buffy coat mononuclear cells were obtained from 100 ml fresh peripheral blood by density gradient centrifugation method using Histopaque 1077 (Sigma, St. Louis, MO) as previously described [8,11]. Buffy coat mononuclear cells were re-suspended in endothelial growth medium (EGM-2) (Cambrex Bioscience, Walkersville, MD) without further subpopulation enrichment procedures, placed into one well of a six well plate coated with type I collagen (BD Biosciences, Bedford, MA) and incubated at 37 °C. Non-adherent cells were removed after 48 h and every second day thereafter. Colonies with cobblestone morphology appeared after 3 weeks in culture. These cells were grown until they formed larger colonies. Colonies were selected, trypsinized, and expanded over several passages.

Human umbilical vein endothelial cells (Cambrex Bioscience) were maintained in EGM-2 medium. Passages 4–8 were used in this study.

2.2. Immunofluorescent staining

Blood outgrowth cells were incubated at 37 °C for 4 h with 10 μg/ml acetylated low density lipoprotein (DiI-Ac-LDL) (Molecular Probes, Eugene, OR) and then were fixed with 3.7% paraformaldehyde for 10 min. After permeabilization with 0.1% Triton X-100 in PBS, rabbit polyclonal anti-human von Willebrand Factor (vWF) primary antibody (Dako Cytomation, Carpinteria, CA) diluted 1:400 in PBS/0.1% BSA was added and incubated for 60 min at room temperature. After two PBS washes, goat anti-rabbit 488 AlexaFluor-conjugated secondary antibody (Molecular Probes) at a 1:1000 dilution in PBS/5% goat serum was added and incubated for 30 min. The samples were rinsed twice with PBS and counter-stained with 1 mg/ml DAPI (Sigma) for 5 min to visualize the cell nucleus.

2.3. Peptide synthesis

All active and control peptide sequences were synthesized using standard Fmoc chemistry on a solid phase peptide synthesizer (Commonwealth Biotech, Inc., Richmond, VA). The peptides were purified by high performance liquid chromatography (HPLC) and chemical purity was confirmed by MALDI-TOF mass spectrometry.

To test the effect of free and immobilized peptides on HBOEC function and binding all experiments were conducted with HBOEC that were quiesced by incubation in endothelial basal medium (EBM-2) (Cambrex Biosciences) containing 0.5% FBS for 16 h. For the migration and tube formation assays as well as for cellular binding experiments, cells were detached with 1 mM ethylene diamine tetraacetic acid (EDTA) (Sigma) at 37 °C for 15 min.

2.4. Cell proliferation assay

The effects of various concentrations of peptides on HBOEC proliferation were examined by counting viable cells as determined by Trypan blue exclusion. Quiescent HBOEC were re-fed with EGM-2 containing free peptides (0, 0.001, 1, and 1000 μmol/L). After 96 h of incubation cells were trypsinized, stained with Trypan blue reagent (Sigma) and viable cells were counted. Proliferation data are presented as fold change compared to the cell numbers before the addition of free peptides.

2.5. Cell migration assay

HBOEC migration was measured by using a 48-well Boyden chamber with 8 μm pore-size filters as previously described [14,15]. EDTA-detached-HBOEC were pre-incubated with peptides (0, 0.001, 1, and 1000 μmol/L) and were seeded at a density of 5000 cells per well in 50 μl migration medium (EBM-2/2% FBS). Recombinant human VEGF isoform 165 (R&D Systems, Minneapolis, MN) was diluted in EBM-2/2% FBS to a concentration 25 ng/ml, and placed in the lower chamber. After incubating at 37 °C for 12 h, cells present at the lower surface of the filter were fixed, stained, and identified in five random fields (20× objective) using a phase contrast microscope. The average number of cells that migrated per condition was calculated. Results are normalized so that the untreated control value is 1.

2.6. Tube formation assay

To analyze capillary tube formation, 40 μl of Matrigel (BD Biosciences) was layered in the wells of a 96-well plate and allowed to solidify at 37 °C for 30 min following the manufacturer's instructions. EDTA-lifted-HBOEC pre-incubated with peptides (0, 0.001, 1, and 1000 μmol/L) were seeded on the Matrigel matrix in a 96-well plate (10 000 cells per well) and cultured at 37 °C for 6 h. Capillary-like structures were examined by phase contrast microscopy, digital images were taken and quantified with ImageJ.

2.7. Spheroid sprouting assay in collagen gels

Sprouting of HBOEC spheroids into collagen matrix was studied as described in Ref. [16]. Briefly, for each collagen gel, 30 spheroids were seeded into 0.7 ml collagen solution in non-adherent 24 well plates with final concentration of rat type I collagen (BD Sciences) of 1.5 mg/ml. Freshly prepared gels were transferred rapidly into a humidified incubator and after solidification 0.25 ml EGM-2 with 10% FBS was added per well. After 36 h gels were examined by phase contrast microscopy and images of the sprouting spheroids were taken.

2.8. Response to VEGF assay

Quiescent HBOEC were re-fed with EBM-2 medium containing 25 ng/ml VEGF, 2%FBS and free peptides (0, 0.001, 1, and 1000 μmol/L). Cells were allowed to proliferate for 96 h and viable cell counts were determined by Trypan blue exclusion. Data were normalized to the initial cell number before the addition of peptides. Statistical significance was determined by comparing to conditions without VEGF.

2.9. Synthesis and characterization of peptide - modified terpolymers

Peptide sequences terminated with cysteine were immobilized to methacrylic terpolymers via one-step chain transfer controlled free radical polymerization as described by Fussell and Cooper [17,18]. The monomers used in the reactions were *n*-hexyl methacrylate (HMA) (Alfa Aesar, Ward Hill, MA), methyl methacrylate (MMA) (ACROS Organics, Pittsburgh, PA), and methacrylic acid (MAA) (ACROS Organics), with 2,2-azobisisobutyronitrile (AIBN) (Aldrich Chemical, Milwaukee, WI) as the initiator. The peptide was added to the monomers after the solvent dimethyl formamide (Aldrich Chemical) was purged with argon. The reaction temperature for the polymerization was 55–60 °C and reactions were carried out overnight. After the reaction was stopped, the solvent was evaporated and the resulting polymer was soaked for 24 h in 1:1 v:v methanol:deionized water mixture to remove unreacted reagents. The purified terpolymer was dried in a vacuum oven at 55 °C for 48 h and then stored in a desiccator. Terpolymer molecular weight was measured by gel permeation chromatography (GPC). The amount of peptide incorporation was determined from amino acid analysis performed by Commonwealth Biotech, Inc. (Richmond, VA).

2.10. Cell binding assay

Peptide-grafted materials were dissolved in acetone and dip-coated on glass coverslips (18 mm, Fisher Scientific, Pittsburg, PA). Vacuum-dried samples were sterilized by immersion in 70% ethanol. After washing with PBS, the coverslips were placed in tissue culture polystyrene plates and blocked with 1% BSA in PBS at 37 °C for 1 h. Wells were rinsed with PBS and peptide-terpolymer constructs were incubated with an HBOEC or HUVEC suspension in serum-free or serum-containing medium at a concentration 20 000 cells/ml. After 2 h of incubation, the medium was aspirated and loosely attached cells were washed off with three changes of PBS. Attached cells were re-fed with EGM-2 containing 10% CCK-8 reagent (Dojindo Molecular Technologies, Gaithersburg, MD) for spectrophotometric determination of relative cell binding. EGM-2 containing 10% CCK-8 was added to three wells without cells to serve as a background control. After incubation at 37 °C for 3 h, 100 μl of solution from each well was transferred into a separate 96-well plate, where the absorbance of the samples against a background control was measured at a wavelength of 450 nm on a Wallac-VICTOR microplate reader (Perkin–Elmer, Wellesley, MA). Cell binding was normalized to attachment of cells to a surface modified with negative control peptide.