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Dual-functioning peptides discovered by phage display increase the magnitude and specificity of BMSC attachment to mineralized biomaterials

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ABSTRACT

Design of biomaterials for cell-based therapies requires presentation of specific physical and chemical cues to cells, analogous to cues provided by native extracellular matrices (ECM). We previously identified a peptide sequence with high affinity towards apatite (VTKHLNQISQSY, VTK) using phage display. The aims of this study were to identify a human MSC-specific peptide sequence through phage display, combine it with the apatite-specific sequence, and verify the specificity of the combined dual-functioning peptide to both apatite and human bone marrow stromal cells. In this study, a combinatorial phage display identified the cell binding sequence (DPIYALSWSGMA, DPI) which was combined with the mineral binding sequence to generate the dual peptide DPI-VTK. DPI-VTK demonstrated significantly greater binding affinity ($1/K_D$) to apatite surfaces compared to VTK, phosphorylated VTK (VTK_{phos}), DPI-VTK_{phos}, RGD-VTK, and peptide-free apatite surfaces ($p < 0.01$), while significantly increasing hBMSC adhesion strength (τ_{50}, $p < 0.01$). MSCs demonstrated significantly greater adhesion strength to DPI-VTK compared to other cell types, while attachment of MC3T3 pre-osteoblasts and murine fibroblasts was limited ($p < 0.01$). MSCs on DPI-VTK coated surfaces also demonstrated increased spreading compared to pre-osteoblasts and fibroblasts. MSCs cultured on DPI-VTK coated apatite films exhibited significantly greater proliferation compared to controls ($p < 0.001$). Moreover, early and late stage osteogenic differentiation markers were elevated on DPI-VTK coated apatite films compared to controls. Taken together, phage display can identify non-obvious cell and material specific peptides to increase human MSC adhesion strength to specific biomaterial surfaces and subsequently increase cell proliferation and differentiation. These new peptides expand biomaterial design methodology for cell-based regeneration of bone defects. This strategy of combining cell and material binding phage display derived peptides is broadly applicable to a variety of systems requiring targeted adhesion of specific cell populations, and may be generalized to the engineering of any adhesion surface.

1. Introduction

Regeneration of large, clinically relevant volumes of tissue *in vivo* using transplanted cells is dependent on having a biomaterial carrier with surface properties that maximize cell attachment and promote cell growth, differentiation and formation of functional extracellular matrix (ECM) [1,2]. Additionally, designing a biomaterial that can promote adhesion of specific cell populations can improve the efficiency of cell based therapies [3]. In the context of bone tissue engineering, inorganic biomaterials, mineralized synthetic or natural polymers, and polymer-mineral composites are used to deliver physical and chemical cues to drive cell adhesion and osteogenesis [4–6]. For example, functionalizing mineralized biomaterials with ECM proteins increases cell attachment, proliferation and differentiation, leading to increased bone healing [7–9].

Peptides derived from the functional domains of ECM proteins can direct stem and progenitor cells toward a bone lineage [3,7,10,11]. Peptide delivery methods involve adsorption, covalent

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immobilization or encapsulation into a biomaterial. The method of delivery and modification of the peptide due to cyclization, post-translational modification or combination with other peptides play important roles in mediating cell responses [12]. Adsorption is the primary mode of peptide delivery to mineral surfaces since covalent immobilization is not possible. Therefore, the accessibility of cell binding domains once the peptide is delivered to a mineral substrate is an important design consideration.

Variability of peptide mediated cell attachment, proliferation, differentiation and tissue regeneration can be linked to both the lack of proper presentation of the peptide to cells and the lack of peptide specificity to certain cell populations [13–15]. Many ECM proteins have multifunctional domains that work in conjunction with one another to present cell instructive sequences to cell surface receptors. For instance, multifunctional ECM-derived or designed peptides such as GTPGPQGIAGQRGVV (P15) and DpSpSEEKFLRRIGRFG (N15)-PRGDS, respectively, demonstrate cell and mineral binding affinity, and impart ostoconductive and ostoinductive cues to adherent osteogenic cells [14,16]. P15, with a sequence similar to one found in the a1 chain of type I collagen, accelerates bone formation in-vivo and has advanced to the clinic in a variety of applications, including healing of periodontal defects, sinus augmentation, alveolar ridge augmentation, fracture healing and lumbar fusion [17–20].

In addition to a cell binding sequence, incorporating a second sequence that tethers the peptide to a biomaterial can mimic these ECM multifunctional domains. Material binding domains have been combined with BMP and VEGF derived peptides to increase adsorption to biomineral surfaces, which in turn can increase cell proliferation, differentiation and drive osteogenesis [9,11,21,22]. Combining cell adhesive peptides with material specific binding domains may allow for greater control of peptide parameters that influence cell recognition. In addition to changing the structure of a peptide, a dual functioning peptide having a material adsorption component can control the presentation of the cell binding sequence to cell surface receptors via both the cell and material binding domains [9,21].

In order to mediate cell specific interactions on apatite surfaces, we used phage display to identify a peptide sequence with high affinity and specificity of binding towards apatite (VTKHLNQISQSY, VTK), especially when phosphorylated [23]. The primary aims of this study were to use phage display to identify a human bone marrow stromal cell (hBMSC) specific sequence, combine it with VTK and measure apatite binding affinity, hBMSC adhesion strength, and specificity to hBMSCs when the apatite and cell-specific peptides are combined into a dual functioning peptide.

2. Materials and methods

2.1. Biomaterial preparation

Hydroxyapatite disks (HAP) for the phage display experiments (10 mm diameter x 4 mm thick) were pressed from powder (Plasma Biotal Ltd. P220) at 1 metric ton for 1 min and sintered at 1350°C for 5 h (heating rate of 10 °C/min). Biomimetic apatite films with a non-stoichiometric apatite surface similar to the inorganic bone micro-environment were used to characterize cell attachment. Apatite films were prepared by immersing 85:15 polylactic-co-glycolic acid (PLGA, Alkermes) thin films in a simulated body fluid to precipitate carbonate substituted apatite with plate like nanofeatures. A 5 w/v% PLGA-chloroform solution was cast on 15 mm diameter glass slides. The PLGA films were etched in 0.5 M NaOH and immersed in modified simulated body fluid (mSBF) for 5 days at 37°C with fluid changes every 24 h. The mSBF was made by dissolving the following reagents in Millipore water at 25°C and titrating to pH 6.8 using NaOH: 141 mM NaCl, 4.0 mM KCl, 0.5 mM $MgSO_4$, 1.0 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 5.0 mM $CaCl_2 \cdot 2H_2O$, and 2.0 mM KH_2PO_4.

2.2. Cell sources and culture

MSCs from various sources (human, mouse primary, human iPS), osteoblasts and fibroblasts were used to assess specificity of peptides to cells. Clonally derived human bone marrow stromal cells (hBMSC) were a generous gift from the NIH [24,25]. Murine bone marrow stromal cells (mBMSCs) were harvested from femora and tibiae of 5–6 week old female C57/BL6 mice (Jackson Laboratories). All BMSCs were cultured in alpha minimum essential media (α-MEM) (Gibco, #12561) with glutamine containing 20% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin (P/S)) (Gibco, #15140) at 37 °C in a 5% CO2 incubator. Induced pluripotent stem cell derived MSCs (iPS-MSC) were a generous gift from Dr. Paul Krebsbach. iPS-MSCs were cultured in (α-MEM), 20% FBS, antibiotics, 200 mM L-glutamine, and 10 mM non-essential amino acids. MC3T3-E1 and mouse dermal fibroblasts (MDFs) were a gift from Dr. Renny Franceschi. MC3T3-E1 cells and mouse dermal fibroblasts were cultured in alpha minimum essential media (α-MEM), 10% FBS, and antibiotics. All cells were passaged when they reached 80–90% confluence. Media was replaced every 2–3 days.

2.3. Combinatorial phage display and screening for hBMSC specific peptides

Peptide sequences with affinity for hBMSCs were identified by screening the Ph.D.12™ Phage Display Library (New England Biolabs, #E8110S), consisting of 10^9 different phage with 12-mer amino acid linear peptide inserts, against clonally derived hBMSCs (Passage 3–6). 2×10^4 hBMSCs were plated in 2 wells of a 6-well dish and cultured for 6 days in culture media at 37°C and 5% CO_2. After 6 days of culture, the NEB 12-mer peptide library was prescreened against sintered HAP disks prior to introduction to the cells, to preferentially screen for sequences attracted to the cells and not the apatite. A streptavidin control was run in a separate dish per the manufacturer's protocol.

Plated cells in 6-well dishes were rinsed 2× with phosphate buffered saline (PBS, Gibco #10010) and pre-blocked with α-MEM containing 0.1% bovine serum albumin (BSA) without supplements at 37°C and 5% CO_2 for 30 min. The aliquot of phage harvested from the HAP disks was then introduced to the cells ($n = 2$). Non-binding phage were then discarded, and the cells were washed 5× in cold PBS. The phage bound to the cells was eluted with 1 mL of Glycine/HCl, pH 2.2, with 1 mg/mL BSA for 10 min at room temperature while being gently rocked. The eluted phage was collected and neutralized with 1 M Tris-HCl, pH 9.1. Three rounds of panning were performed for each sample.

2.4. RELIC analysis

The bioinformatics tool REceptor LIgand Contacts (RELIC) [26–28] was used to analyze the data from the phage display. The programs DNA2PRO, MOTIF1, and INFO determined peptide translation sequences from DNA code, continuous conserved motifs within the peptide population allowing for conservative substitutions, and information numbers for each peptide reflecting the probability of selecting individual phage by chance, respectively. The phage display experiment on hBMSCs yielded 50 candidate peptides, which were subsequently used in the MOTIF1 and INFO programs. The program MOTIF1 searched for 3-mer and 4-mer continuous conserved motifs. The program INFO was run with