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Dual-functioning phage derived peptides encourage human bone marrow cell specific attachment to mineralized biomaterials

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Abstract

Cell instructive mineralized biomaterials are a promising alternative to conventional auto-, allo-, and xenograft therapies for the reconstruction of critical sized defects. ECM proteins, peptide domains, and functional motifs demonstrating cell and mineral binding activity have been used to improve cell attachment. However, these strategies vary in their tissue regeneration outcomes due to lack of specificity to both regenerative cell populations and the material substrates. In order to mediate cell specific interactions on apatite surfaces, we identified peptide sequences with high affinity towards apatite (VTKHLNQISQSY, VTK) and clonally derived human bone marrow stromal cells (DPIYALSWGMA, DPI) using phage display[1–3]. The primary aims of this study were to measure apatite binding affinity, human bone marrow stromal cell (hBMSC) adhesion strength, and peptide specificity to hBMSCs when the apatite and cell specific peptides are combined into a dual functioning peptide. To assess binding affinity to hydroxyapatite (HA), binding isotherms were constructed and peptide binding affinity (K_d) determined. HBMSC, MC3T3 and mouse dermal fibroblast (MDF) adhesion strength on biomimetic apatite functionalized with single and dual-functioning peptide sequences were evaluated using a centrifugation assay. DPI-VTK had the highest binding strength towards hBMSCs ($p < 0.01$). DPI-VTK, while promoting strong initial attachment to hBMSCs, did not encourage strong adhesions to MC3T3s or fibroblasts ($p < 0.01$). Taken together, phage display is a promising strategy to identify preferential cell and material binding peptide sequences that can tether specific cell populations onto specific biomaterial chemistries.

Keywords

Biomimetic apatite; tissue regeneration; Langmuir isotherms; binding affinity; centrifugation assay

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Declaration of Interest

There are no outside contributors to the completion of this work or any known conflicts of interest.

Introduction

Current grafting therapies for skeletal defects can result in complications such as donor site morbidity and immunogenicity, while being limited by availability of tissues. Cell based tissue regenerative therapies are an alternative that addresses these unmet clinical needs. Cell based strategies often require a biomaterial carrier that provides physical support and bridging of the void space, along with the ability to direct cell fate.

Many extracellular matrix proteins and peptide derivatives have been used to improve cell attachment to mineralized biomaterials. For instance, known mineral binding peptide domains and motifs in bone, dentin and enamel ECM proteins as well as mineral binding domains combined with cell binding sequences have been used to improve cell attachment to apatite surfaces[4]. In order to mediate cell specific interactions on apatite surfaces, we identified peptide sequences with high affinity towards apatite (VTKHLNQISQSY, VTK) and clonally derived human bone marrow stromal cells (hBMSC) (DPIYALSWGMA, DPI) using phage display[1, 2] and have shown increases in the magnitude and specificity of binding to apatite with VTK [2], especially when phosphorylated [3]. The primary aims of this study are to 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.

Materials and Methods

Peptide adsorption to HA

Bone-like mineral synthesis, phage display methods, peptide synthesis and a list of peptide sequences are described in the supplement methods. Phage derived single and dual-functioning peptides and control peptides were incubated on HA powder (Sigma Aldrich) at 37°C in Trizma buffer (pH 7.5) for 3 hours in a 96-well Millipore filter plate. Unbound peptide solution was filtered into a fresh 96 well plate and unbound peptide amount was determined using standards. Bulk peptide concentrations tested ranged from 0–2000 µg/mL. Peptides demonstrate no affinity towards tissue culture polystyrene surfaces and therefore no peptide was bound to the plate walls which could cause an overestimate of peptide binding to mineral [2]. To assess affinity to apatite, Langmuir isotherms of bulk versus bound peptide were constructed to determine binding affinity (K_1). Two experiments were done each with 3 replicates and two technical replicates.

Cell adhesion strength and specificity on peptide-coated biomimetic apatite films

hBMSC, MC3T3 and mouse dermal fibroblasts (MDF) were cultured in α -MEM with glutamine, 20%FBS, 1% Penicillin/streptomycin. Biomimetic apatite films were incubated in ddH₂O overnight to remove excess salts and incubated in TRIZMA buffer (pH 7.5)[3]. Mineralized films were separated from the underlying coverglass and attached to the bottom of 24 well plates with sticky tabs. Films were subsequently incubated in 100 µg/mL of peptide solution for 3 hrs, washed and blocked with 1% denatured bovine serum albumin to reduce non-specific cell attachment to biomimetic apatite. Peptide coated films, no peptide controls and standards were incubated with 75k cells/mL in each well for 3hrs at 37°C and

5% CO₂ in serum-free media. Cells were subsequently washed, wells were filled with PBS, inverted, sealed and centrifuged with detachment forces of 0–10⁻⁵ dynes using an Eppendorf 5810r centrifuge. Detection limits for the detachment force assay were optimized using no peptide control films, FITC-BSA coated films, and biochemical staining methods. At detachment forces above 10⁻⁵ dynes, mineral stability and FITC-BSA adsorption were compromised. Therefore the detection limits of the assay were constrained from 0–10⁻⁵ dynes. Detached cells were removed, and adherent cell fractions were determined using the WST-1 assay (Clontech Laboratories, Inc.), and half-cell detachment forces (τ_{50}) were determined by fitting sigmoidal curves using Microsoft Excel's solver with the Boltzmann equation.

Statistical Methods

Single factor ANOVA was used to determine statistical differences amongst peptide groups for both apatite isotherms and cell adhesion strength assays.

Results

Peptide adsorption to HA

The phage display yielded the experimental peptides used in this study (see supplement for methods and sequences), Binding affinities of mineral binding peptides VTK and VTK_{phos} were significantly greater than the binding affinities of the cell specific peptides, and VTK_{phos} demonstrated a higher K₁ than VTK (Fig. 1a). The dual-functioning phage derived peptides DPI-VTK, DPI-VTK_{phos}, and cell binding control peptide RGD-VTK all demonstrated significantly higher binding affinities than the single peptides ($p < 0.01$). Interestingly, DPI-VTK had a significantly higher K₁ than DPI-VTK_{phos} ($p < 0.01$). Although the acidic residues in E7 bind strongly with the cationic components of HA, the binding affinity of RGD-E7 was lower than predominantly charge neutral RGD-VTK and DPI-VTK ($p < 0.01$).

Cell adhesion strength and specificity on peptide-coated biomimetic apatite films

Dual peptides RGD-VTK and RGD-E7 showed the highest initial cell attachment when no force was applied (Fig. 1 supplement; $p < 0.01$). However as detachment forces were applied, larger cell fractions were adherent to DPI-VTK and consequently τ_{50} for the DPI-VTK sequence was significantly higher than the rest of the peptides (Fig. 2a $p < 0.01$). DPI-VTK bound more strongly to hBMSCs, compared to pre-osteoblasts and fibroblasts (Fig. 2b $p < 0.01$). Although, RGD-VTK bound weakly to hBMSCs, it promoted stronger adhesions with MC3T3s and MDFs ($p < 0.01$).

Discussion

Peptide adsorption to HA

Dual peptides improved binding to HA compared to single peptides, indicating that both cell and mineral binding components contribute to K₁. Phosphorylation improves peptide-mineral interaction in extracellular matrix proteins [4, 5]. Although VTK_{phos} had a higher binding affinity than VTK, potential peptide conformational differences in DPI-VTK

without the charged mineral binding tail of VTK_{phos} could be contributing to differences in apatite binding affinity, also indicating that the cell binding sequence interacts with the mineral. Moreover, RGD-E7 with an acidic mineral binding motif demonstrated the lowest binding affinity amongst the dual functioning peptides. The K_1 values for RGD-E7 are consistent with previous studies using similar materials and assay conditions [6]. Taken together, peptide sequence and conformation contribute significantly to apatite binding affinity. This is in accordance with data indicating that the sequence of VTK plays a critical role in apatite binding[3].

Cell adhesion strength and specificity on peptide-coated biomimetic apatite films

The half-cell detachment force is a measure of the force at which 50% of the initially bound cell population becomes detached. Detachment forces relate to how specifically and strongly cells attach to the substrate. Since the adhesion timeframe in serum-free media allows for initial attachment only, the detachment force correlates with the average number of peptide-cell ligand interactions over a population of cells[7]. The dual-functioning phage derived peptide DPI-VTK demonstrated the highest hBMSC adhesion strength. VTK and VTK_{phos} sequences did not exhibit the same level of adhesion strength, indicating that the cell adhesion strength of DPI-VTK is largely driven by the interaction with the DPI peptide. Moreover, weaker attachment of DPI-VTK_{phos} to hBMSC compared to DPI-VTK indicates that phosphorylating the mineral sequence does not improve the presentation of DPI sequence to the cell. Favorable apatite binding of DPI-VTK compared to DPI-VTK_{phos}, and favorable hBMSC binding of DPI-VTK compared to DPI-VTK_{phos} indicates an involvement of both cell and mineral binding components of the bi-functional peptides in both mineral and/or cell binding either directly or indirectly. For instance, adding DPI to the VTK sequence could be contributing to conformational changes to both mineral and cell binding sequences that enhance mineral binding affinity. Once bound, association with the biomimetic apatite can cause another structural change to both DPI and VTK resulting in increased or decreased affinity to cell binding targets. The differences in cell and mineral specificity of DPI-VTK and DPI-VTK_{phos} could arise from these conformational differences in the solution and bound states. Future studies will consider the contribution of conformational differences amongst bi-functional peptide linkers to explore the mechanism of interaction contributing to both cell and mineral specificity.

Although RGD-VTK and RGD-E7 peptides demonstrated high initial cell binding, the weaker adhesion strength is indicative of weak association between hBMSC binding targets and RGD when presented with E7 and VTK sequences. This could result from the selectivity of mineral bound RGD-VTK to a particular integrin subunit that is more highly expressed in MC3T3-E1 and fibroblasts. The adhesion strength of MC3T3-E1 cells and MDF to DPI-VTK was significantly less than to hBMSCs. This is indicative of DPI-VTK selectivity towards an hBMSC specific target, confirming the value of phage display as a strategy to identify cell specific peptides for use in tissue engineering.

Conclusions

Collectively, the data suggest that the individual peptides contribute to both functionalities of the dual functioning peptide. However, the mineral binding sequence binds favorably to apatite and the cell binding sequence binds strongly and specifically to hBMSCs. The sequence plays a significant role in the preferential binding of peptides to apatite or cell surfaces. Phage display is a promising strategy to identify apatite and cell specific sequences that can subsequently be combined to encourage cell specific attachment to mineralized biomaterials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Segvich, SJ. Design of Peptides with Targeted Apatite and Human Bone Marrow Stromal Cell Adhesion for Bone Tissue Engineering. University of Michigan; 2008.
2. Segvich SJ, Smith HC, Kohn DH. The adsorption of preferential binding peptides to apatite-based materials. *Biomaterials*. 2009; 30:1287–1298. [PubMed: 19095299]
3. Addison WN, Miller SJ, Ramaswamy J, Mansouri A, Kohn DH, McKee MD. Phosphorylation-dependent mineral-type specificity for apatite-binding peptide sequences. *Biomaterials*. 2010 Dec; 31(36):9422–9430. [PubMed: 20943264]
4. Hennessy KM, Clem WC, Phipps MC, Sawyer AA, Shaikh FM, Bellis SL. The effect of RGD peptides on osseointegration of hydroxyapatite biomaterials. *Biomaterials*. 2008 Jul; 29(21):3075–3083. [PubMed: 18440064]
5. Gilbert M, Shaw WJ, Long JR, Nelson K, Drobny GP, Giachelli CM, Stayton PS. Chimeric peptides of statherin and osteopontin that bind hydroxyapatite and mediate cell adhesion. *J Biol Chem*. 2000 May; 275(21):16213–16218. [PubMed: 10748043]
6. Fujisawa R, Mizuno M, Nodasaka Y, Kuboki Y. Attachment of osteoblastic cells to hydroxyapatite crystals by a synthetic peptide (Glu7-Pro-Arg-Gly-Asp-Thr) containing two functional sequences of bone sialoprotein. *Matrix Biol*. 1997 Apr; 16(1):21–28. [PubMed: 9181551]
7. McClay DR, Hertzler PL. Quantitative measurement of cell adhesion using centrifugal force. Chapter 9. *Curr Protoc Cell Biol*/ ed Juan S Bonifacino. 2001 May. Unit 9.2.

Figure 1. Single and dual-functioning peptide monolayer adsorption (K_1) on HA particles (** indicates significant difference from all groups, * indicates significant different from DPI-VTK and unstarred groups, n = 6)

Figure 2.

a Half-cell detachment forces of hBMSCs on single and dual-functioning peptide coated biomimetic apatite (*indicates significant difference vs. other peptide groups, n=6)

b Half-cell detachment forces on dual-peptide coated apatite films (* indicates significant difference vs. other cell groups, n=6)