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Inhibition of osteoblast mineralization by phosphorylated phage-derived apatite-specific peptide

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ABSTRACT

Functionalization of biomaterials with material- and cell-specific peptide sequences allows for better control of their surface properties and communication with the surrounding environment. Using a combinatorial phage display approach, we previously identified the peptide VTKHLNQISQSY (VTK) with specific affinity to biomimetic apatite. Phosphorylation of the serine residues of the peptide (pVTK) caused a significant increase in binding to apatite, as well as a dose-dependent inhibition of osteoblast mineralization. In this study, we investigated the mechanisms behind pVTK mediated inhibition of mineralization using MC3T3 cells and testing the hypothesis that mineralization is inhibited via alteration of the Enpp1–TNAP–Ank axis. Inhibition of mineralization was not due to disruption of collagen deposition or calcium chelation by the negatively charged pVTK. The timing of peptide administration was important in inhibiting mineralization – pVTK had a greater effect at later stages of osteogenic differentiation (days 7–12 of culture corresponding to matrix maturation and mineralization), and could prevent progression of mineralization once it had started. pVTK treatment resulted in a significant decrease in ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1) enzyme activity and gene expression. The expression of ankylosis protein (Ank), osteopontin (OPN) and Pit-1 genes was also significantly reduced with peptide treatment, while tissue non-specific alkaline phosphatase (TNAP), bone sialoprotein (BSP), and Runx2 gene expression was significantly higher. The ability of pVTK to inhibit mineralization can potentially be translated into therapeutics against pathological calcification seen in cardiovascular disease, osteoarthritis or craniosynostosis, or be used to prevent failure of biomaterials due to calcification, such as bioprosthetic heart valves.

1. Introduction

Biomaterials should be able to communicate with their microenvironment, to encourage attachment of specific populations of cells onto their surfaces, and eventually regenerate functional tissue. Surface modification of biomaterials with cell-specific biological factors can provide the signals required to initiate cell adhesion. One approach to controlling biomaterial surface properties is via functionalization with cell- and material-specific peptide sequences, which can serve as anchors between a specific cell population and a specific material chemistry. Short synthetic peptides (9–30 amino acids in length) are advantageous for this application since they can be designed to be non-immunogenic, as well as more specific than full length proteins (containing multifunctional domains). Further, short peptides are easier and less expensive to synthesize, and are less likely to change conformation since they are not as prone to forming secondary/tertiary structures [1].

Peptide functionalized apatite-based biomaterials are useful in bone tissue engineering as they mimic aspects of the organic/inorganic hybrid composition of bone (primarily comprised of organic collagen and non-collagenous proteins, and inorganic hydroxyapatite). Traditionally, peptide sequences for bone regeneration have been derived from known bone-binding motifs found in non-collagenous proteins, often containing chains of acidic aspartate

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and glutamate residues. For example, the peptide EEEEEEEPRGDT (E7PRGDT), derived from bone sialoprotein (BSP), consists of a polyglutamic hydroxyapatite binding domain and the ubiquitous RGD cell binding domain, and mediates osteoblast attachment to hydroxyapatite and subsequent osteogenesis [2,3]. The fusion peptide N15-PGRGDS, comprising a hydroxyapatite binding sequence (N15) derived from statherin and the RGD cell binding sequence from osteopontin (OPN), facilitates dose-dependent attachment of cells to hydroxyapatite [4]. Modular peptides have also been designed that contain apatite binding sequences derived from osteocalcin (OCN) and growth factor sequences derived from bone morphogenetic protein 2 (BMP2) [5]. These peptides modulate cell binding to apatite, and peptide-coated implants stimulate greater bone formation and ingrowth compared to untreated controls in a sheep bone-implant gap model [6].

A novel way of discovering unique material-specific peptides is through the use of phage display, which involves panning bacteriophage libraries expressing ~10^9 sequences against a material of interest. Using this approach, along with computational modeling and ELISA, we identified the apatite-specific 12-mer peptide VTKHLNQISQSY (VTK) [7,8]. However, a disadvantage of phage libraries is the inability of bacteriophage to incorporate peptide post translational modifications, such as phosphorylation of serine, threonine and tyrosine residues, which are particularly important in the regulation of biomineralization [9,10]. To overcome this drawback and further characterize the mineral-binding VTK sequence, we phosphorylated the serine residues on VTK (pVTK) and measured a 10-fold increase in adsorption to synthetic biomimetic apatite, and significantly higher binding affinity of pVTK compared to non-phosphorylated VTK [11].

Additionally, pVTK caused a dose dependent inhibition of mineralization in MC3T3 pre-osteoblastic cells, with minimal cytotoxic effects. Although VTK and pVTK bind to both synthetic and cell-secreted mineral, the relative contribution of peptide sequence and charge is different between the two forms of apatite. Phosphorylation and net charge were more important in peptide binding to synthetic mineral (scrambling the sequence had no effect on binding to biomimetic mineral), whereas peptide sequence and phosphorylation/charge were equally important in inhibiting biological mineralization (scrambling pVTK resulted in 30% less inhibition compared to pVTK) [11]. Understanding how the phosphorylated peptide interacts with mineral and/or cells to inhibit mineralization could enable the application of pVTK in the treatment of pathological mineralization.

Development of therapeutics against pathological calcification is dependent on advancing knowledge of mechanisms of mineralization. Briefly, mineralization is a tightly regulated process determined by the concentrations and properties (including net charge, charge distribution or number of acidic/phosphorylated amino acid residues) of extracellular matrix proteins and other promoter and inhibitor molecules. Inorganic extracellular pyrophosphate (PPi) is a natural inhibitor of mineralization (at micromolar concentrations) and is formed as a by-product of several metabolic reactions. The concentration of extracellular PPi is regulated mainly by the action of tissue non-specific alkaline phosphatase (TNAP), ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1) and the progressive ankylosis protein (Ank). Enpp1 and Ank help inhibit calcification by increasing the concentration of extracellular PPi – Enpp1 cleaves ATP to release PPi and Ank transports PPi from within to outside the cell [12,13]. TNAP decreases PPi levels by cleaving PPi to generate phosphate (Pi), thus promoting calcification [14,15]. The actions of TNAP, Enpp1 and Ank maintain the PPi/Pi ratio, which has a direct consequence on mineralization – high PPi/Pi ratios inhibit, while low PPi/Pi ratios promote mineralization. The presence of Enpp1, Ank, TNAP, as well as other osteogenic markers in pathologic mineral deposits [16,17], indicate similarities between physiological (bone) and pathological calcification mechanisms, providing new opportunities for development of therapeutics against pathological calcification.

In this study, we hypothesized that pVTK inhibits MC3T3 mineralization via alteration of the Enpp1–TNAP–Ank axis. We performed assays at the gene and protein-levels to test the effect of peptide treatment on mineralization, collagen deposition, Enpp1 and TNAP enzyme activity, and osteogenic differentiation in MC3T3 cells. The effect of peptide administration on mineralization at various stages of osteogenic differentiation, and the ability to inhibit progression of mineralization in cultures after the initiation of mineralization were also investigated.

2. Materials and methods

2.1. Peptide synthesis

The following peptides were used: pVTK (VTKHLNQI(pS)Q(pS)Y; pS = phosphoserine), FITC-pVTK (FITC-VTKHLNQI(pS)Q(pS)Y) and FITC-E7-RGD (FITC-EEEEEEEPRGDT). Peptides were synthesized using Fmoc solid-phase chemistry according to standard peptide synthesis procedures and characterized as having >90% purity by high-performance liquid chromatography (University of Michigan Proteomics & Peptide Synthesis Core). Phosphorylation at specific serine residues was achieved using preformed, protected phosphoserine amino acids. For experiments involving fluorescence, peptides were labeled on resin before cleavage using 5-(and-6)-carboxyfluorescein succinimidyl ester (Molecular Probes C-1311). The peptides were dissolved in ddH_2O and aliquots were frozen at −20 °C until ready to use in experiments.

2.2. Cell culture and overview of experimental design

MC3T3-E1 (subclone 14) murine calvarial pre-osteoblasts were maintained in alpha minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (growth medium) at 37 °C and 5% CO_2 in a humidified incubator. For all experiments, cells were seeded at a density of 10,000 cells/cm^2 (48 well plates, Corning Costar) and allowed to attach overnight in growth medium. Cells were then differentiated with osteogenic medium (growth medium supplemented with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid) with or without 300 μM pVTK peptide and cultured for up to 12 days. Media was replaced every 2 days. For analysis of calcium, media was collected every two days during the media change and frozen at −80 °C until assayed.

To understand the effect of pVTK on differentiation and mineralization, gene expression of Enpp1, TNAP, Runx2, bone sialoprotein, osteocalcin, osteopontin, Ank and Pit-1 was measured at days 3, 7, 10 and 12. The expression of collagen I was also measured after 12 days of peptide treatment. Protein expression (Runx2, OPN) and enzyme activity (Enpp1, TNAP) were also measured to confirm the gene-level data. To further determine the inhibitory effects of the peptide at different stages of osteogenic differentiation and mineralization, cells were cultured for 12 days with pVTK added at days 1–6 only (corresponding to cell proliferation and matrix production) or days 6–12 only (corresponding to matrix maturation and mineralization), and compared to untreated controls and cells cultured with the peptide for the entire 12 days. To assess the effect of peptide on mineralizing cell layers, cells were cultured in osteogenic media only until mineralization started on day 10, after which pVTK was added and cells were cultured until day 14.