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Identification of putative bone anabolic peptides targeting adherent plasma membrane

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ABSTRACT

Bone matrix provides unknown essential cues for osteoblast lineage cells to develop, grow, repair and remodel bones via adherent plasma membrane. Because of its tight sealing with bone matrix in vivo and culture surface in vitro as well, the adherent plasma membrane has been unveiled target of investigation to date. Herein, we report a new approach to explore the adherence plasma membrane of osteoblasts with biofunctional peptide candidates in a bacterial peptide library. To accomplish this, human osteoblast like hFOB 1.19 cells were cultured on porous filter with 8 μm pore through which bacterial peptides were allowed to meet the membrane for affinity selection. The affinity-selected peptides were coated on culture plate to further evaluate their influence on osteoblastic cell adhesion, as well as expressions of osteoblast differentiation markers, alkaline phosphatase and osteocalcin. Finally, the serial screenings identified two prominent active peptides that enhanced the differentiation markers nearly to the same level as a control peptide of bone morphogenetic protein-2. Osteogenic activity is expected for the peptides when immobilized on bone implant surface.

1. Introduction

The extracellular matrix (ECM) not only provides structural support for cells engaged in maintenance and remodeling of tissue structure, but it also allows the signaling necessary for those cells to differentiate or elicit biological function [1–3]. Thus, the impact of ECM on cellular physiology with respect to tissue and organ structure has engendered many studies, in particular the investigation of ECM biomimetic agents for tissue regenerative therapy [4]. It is also true that bone matrix (BM) contains critical bioactive proteins that induce the recruitment of osteoblast precursor cells and promote their differentiation into mature form [5,6]. Replication and differentiation of osteoblast lineage cells are conducted by mutual interaction between adherent plasma membrane receptors and protein epitopes exposed on the surface of BM. Multiple BM proteins, such as the family of bone morphogenetic proteins (BMPs), have been shown to possess bone anabolic function on osteoblast precursors. However, clinical application of bone anabolic proteins, in particular, those represented by BMPs, is still significantly limited by short half-life and/or low retention rate in the bone and its surrounding tissue. The cost of generating recombinant protein also poses a barrier against the development of therapeutic modalities utilizing bone anabolic proteins. Those drawbacks can be addressed with chemical and structural modifications of peptides [7,8]. Furthermore, peptide libraries generated by either phage or bacterial display system provide hundreds of millions of random peptide sequences, thus offering an ideal platform for ligand screening.

Whole plasma membrane of suspension cells [9–11] and apical plasma membrane of adherent cells [12,13] offer good targets for peptide ligands. On the other hand, the adherent plasma membrane appears not to have been targeted for a screening even though it is absolutely critical for the recruitment and differentiation of epithelial and mesenchymal cells [2,14]. This is attributed to the simple fact that the basal side plasma membrane adheres tightly to a solid culture surface, and this renders interaction studies challenging. In order to address such limitation, we designed a new approach that allowed serial

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screening of peptide library against the outer surface of plasma membrane where osteoblasts adhere to culture surface in vitro. In this manner, we identified two novel peptides that stimulated osteoblastic functions from the outer surface of adherent plasma membrane.

2. Materials and methods

2.1. Osteoblast cell cultures

hFOB 1.19 cells (CRL-11372) and MG-63 (CRL-1427) cells were purchased from ATCC (Manassas, VA, USA). hFOB 1.19 cells were maintained in a 1:1 mixture of Ham's F12 and DMEM supplemented with 2.5 mM glutamine, 0.3 mg/ml of G418 and 10% FBS and without phenol red at 34 °C in humidified 5% CO_2-95% air. In functional assays, hFOB 1.19 cells were cultured at 37 °C in the absence of G418. MG-63 cells were cultured at 37 °C in Eagle's MEM supplemented with 10% FBS.

2.2. Affinity selection of peptide against the adherent plasma membrane

All following procedures were done under sterile condition. Bacterial peptide display library (FliTrx, Invitrogen, CA, USA) was used for biopanning in human preosteoblastic hFOB 1.19 cells according to the manufacturers' instructions. Briefly, bacteria were cultured in IMC medium for growth, and in tryptophan-supplemented IMC for induction of conditional peptide expression. IMC medium was composed of M9 Salts, 0.2% casamino acids, 0.5% glucose, 1 mM $MgCl_2$, and 100 μg/ml ampicillin. FliTrx expressed dodecamer cyclic peptides were displayed on a flagellin-thioredoxin scaffold (FliTrx-peptide) on the bacterial surface. FliTrx-peptides were physically cleavable at the flagellin by vortexing. The biopanning procedure was followed according to the original instructions except that the adherent plasma membrane of cultured hFOB 1.19 cells was used as bait instead of an immobilized protein on a plastic dish. Briefly, hFOB 1.19 cells were first grown to confluence in a culture insert with 8-μm pore filter (Millipore, Billerica, MA, USA). After hFOB 1.19 cells reached confluence, the side-wall of culture insert was wrapped tightly with parafilm to extend an incubation chamber to the opposite side of the cell compartment for biopanning. The culture insert was then flipped upside-down in a 25 ml beaker, and a 2 ml suspension containing 2 billion peptide-induced bacterial cells in PBS containing 1% BSA was applied to the extended chamber. After 15 min of incubation with a gentle cradle at room temperature, unbound cells were washed off five times with PBS, and the remaining bound bacteria were resuspended in 4 ml of IMC medium. Bacteria were spun down for 10 min at 3000 rpm and finally resuspended in 200 μl of IMC medium. Half-suspension was stored as 20% glycerol stock and the rest was titrated at a five-fold dilution series. Hundred-μl of the titrations were streaked on RMG agar plates (10 cm in diameter) to obtain approximately 200 colonies in a dish. Ninety-four clones were randomly picked and transferred to a v-bottom 96-well storage plate (polypropylene, Corning, USA). The primary hit bacterial clones were amplified and duplicated. One was stored in IMC containing 20% glycerol at −80 °C for later DNA sequencing. The other plate was incubated to amplify bacteria until OD600 reached 0.5, and then induced FliTrx-peptides in tryptophan-supplemented IMC for 6 h. Induced FliTrx-peptides were cleaved from bacterial cells by vortex and recovered in the supernatant by centrifugation at 3000 x*g* for 10 min. The peptide suspension was incubated at 60 °C for 30 min in the presence of heat stable kanamycin at 50 μg/mL to kill possible contamination of bacteria.

2.3. Functional evaluations of FliTrx-peptides

2.3.1. Cell adhesion assay

FliTrx-peptides, native flagellin, and poly-L-lysine were coated to non-tissue culture low binding microtiter plates (Corning, MA, USA) at 1 μg/100 μl/well in PBS, and washed three times with PBS-0.1% Tween 20. Unoccupied sites were blocked with 1% BSA in PBS. hFOB 1.19 cells were trypsinized, washed twice with PBS, and resuspended in the culture medium containing 10% FBS to be 3×10^5 cells/mL. Hundred-μl of the cell suspension was added to the coated wells, incubated at 37 °C for 4 h, and then gently rinsed with the culture medium. The remaining cell number was evaluated by CellTiter-Glo Luminescent Cell Viability assay (Promega, WI, USA).

2.3.2. Cellular ALPase activity and OCN release

hFOB 1.19 cells were seeded to the FliTrx-peptide and control coated wells at 1.5×10^4 cells/well. Cellular alkaline phosphatase (ALPase) activity and osteocalcin (OCN) release in the culture medium were measured on day 3 by ALPase fluorometric assay (Biovision, CA, USA) and on day 6 by OCN ELISA kit (R&D systems, MN, USA), respectively. For OCN expression, the culture medium was replaced once on day 3. Double strand DNA was measured by PicoGreen dsDNA assay kit (Life technologies, NY, USA) to normalize ALPase activity and OCN expression.

2.4. Effects of synthetic peptides

Bacterial clones in glycerol stocks corresponding to the peptides C7 and H10 that showed robust inductions of ALPase activity and OCN expression were grown in RM medium for plasmid DNA sequencing. RM medium contained 1X M9 salts, 2% casamino acids, 1% glycerol, 1 mM $MgCl_2$, and 100 μg/ml ampicillin. Plasmid DNA were isolated from three independent clones for each peptide and sequenced to determine amino acid sequences. Consensus sequences were obtained for C7 (GKIHRHRGQAVE) and H10 (ESHCLLGISCVL) (US patent #8,815,807). These two peptides were chemically synthesized as circular form (head to tail) with biotin-conjugated on N-terminus at a contract research organization (Genscript, NJ, USA). Biotinylated-linear BMP-2 (aa73~92: KIPKASSVPTELSAISTLYL) [15] was also synthesized as control. Two-nmole of biotinylated-peptides was added to streptavidin-coated well (biotin-binding capacity: 125 pmole/well; Thermo Scientific, MA, USA) to ensure the saturation of the biotin-binding sites. hFOB 1.19 and MG-63 cells were seeded to the peptide-coated wells at 1.5×10^4 cells/well, cultured, and assayed for ALPase activity and OCN release as previously described.

2.5. Statistical analysis

Bar charts show mean ± SD of three independent experiments. Statistical analyses were done by one-way ANOVA and Fisher's PLSD. P-value of <0.05 was considered statistically significant.

3. Results

3.1. Affinity selection of peptides against adherent plasma membrane

Biopanning was carried out in completely confluent monolayer of hFOB 1.19 cells where trypan blue did not permeate from the apical side to the basal side and vice versa (data not shown). After single round of biopanning approximately 200 single colonies were obtained from the first 5-fold titration of the bacterial suspension. Ninety-four clones were picked for the next cell adhesion assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**100**	**153**	122	95	107	137	96	140	124	149	118	159
B	98	105	156	119	121	161	126	131	215	103	109	115
C	119	99	102	111	127	112	238	176	106	194	187	83
D	104	213	102	113	108	101	147	103	98	92	193	86
E	104	97	125	114	176	159	103	174	125	88	90	83
F	101	104	170	100	135	223	156	135	95	133	85	100
G	110	97	93	108	120	119	124	183	99	173	140	85
H	148	111	146	218	139	171	168	169	243	205	124	103

Fig. 1. Effects of FliTrx-peptides on cell adhesion in hFOB 1.19 cells. Assay wells were coated with A1: nativeFli (native flagellin), A2: poly-L (poly-L-lysine), and A3 ~ H12: affinity-selected peptides. Values indicate mean percentage relative to A1-nativeFli control (n = 3).

3.2. Functional assays of FliTrx-peptides

3.2.1. Cell adhesion

The affinity positive FliTrx peptides were immobilized to non-tissue culture treated-wells to see if they supported cell adhesion when immobilized to solid support assuming clinical roles such as coating on dental implant and bone scaffold. Native flagellin collected from DH5α *E. coli* strain, which was the vehicle in the FliTrx system, was used as blank reference. Poly-L-lysine was used as a positive reference. Most peptides showed higher adhesion activity than native flagellin (Fig 1, well-A1), and twenty-four peptides (highlighted wells in gray) supported cell adhesion greater than the positive reference of poly-L-lysine (well-A2). The top 10 FliTrx-peptides (highlighted in bold white in gray) were further tested for ALPase activity and OCN expression.

3.2.2. ALPase activity and OCN expression

FliTrx-peptides H9, C7, F6, H4, D2, B9, H10, B8, C11, and D11 significantly increased ALPase activity as compared to native flagellin (Fig 2A). These peptides, except B9, also increased OCN release significantly (Fig. 2B). Since exogenous peptide-free native flagellin was significantly less active, the effects were most likely due to the effect of the peptides. The C7 and H10 peptides showed especially prominent effects that were significantly greater than the other peptides and the controls. In order to confirm the single effect of peptide without FliTrx scaffold, DNA sequences of C7 and H10 peptides were determined for chemical peptide synthesis.

3.3. Effects of chemically synthesized peptides on ALPase activity, OCN secretion and calcification

Since FliTrx system expressed cyclic peptides due to disulfide bridge between C- and N-terminus, C7 and H10 peptides were also synthesized as cyclic form (C7: GKIHRHRGQAVE, H10: ESHCLL-GISCVL; both head-to-tail circularized). Head-to-tail circularization was taken to prevent peptide multimer formation that should not occur in FliTrx peptide display. Linear peptide of BMP-2 (KIP-KASSVPTELSAISTLYL), which was previously shown to induce ALPase, OCN, and calcification in vitro and vivo [15–17], was also synthesized and used as a positive control. In order to coat assay plates with the different peptides at similar degree biotin was linked to the peptides. Streptavidin-coated assay wells were saturated with the biotinylated peptides for ALPase activity (Fig. 3A) and OCN expression (Fig. 3B) in hFOB 1.19 and MG-63 undifferentiated osteoblast like cells. Both C7 and H10 peptides showed robust induction of ALPase and OCN at levels comparable to that of the positive control of BMP-2 peptide. These results clearly

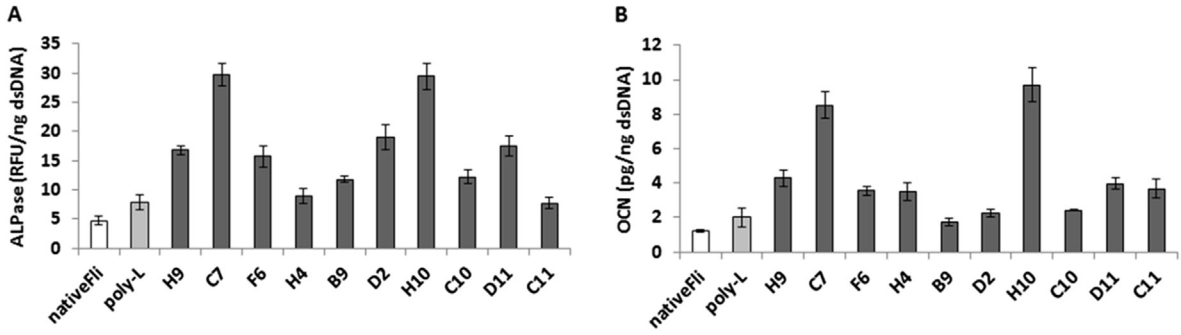

Fig. 2. Effects of FliTrx-peptides on cellular ALPase activity and OCN release in hFOB 1.19 cells. (A) Cellular ALPase activity. (B) OCN release in culture medium. nativeFli: native flagellin; poly-L: poly-L-lysine. Bars indicate mean + SD, n = 3.

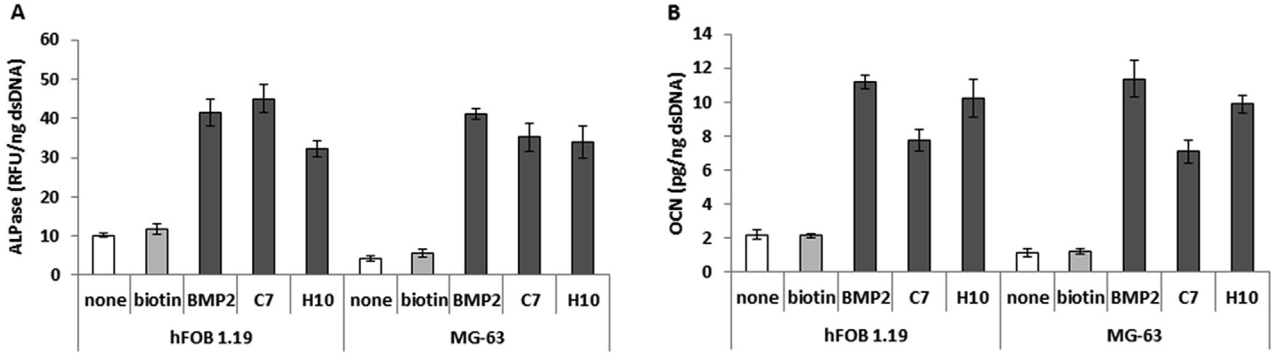

Fig. 3. Effects of synthetic peptides on cellular ALPase activity and OCN release in hFOB 1.19 and MG-63 cells. (A) Cellular ALPase activity. (B) OCN release in culture medium. Bars indicate mean + SD, n = 3.

demonstrated that peptide displayed in C7 and H10, but not in FliTrx scaffold, are responsible for the induction of signals that promote expression of ALPase and OCN.

4. Discussion

Standard biopanning repeats successive rounds of binding, washing and amplification steps to enrich clones expressing high affinity peptides against a single target protein such as an antibody, ligand or receptor. On the other hand, our technique targets multiple binding pairs simultaneously between peptides as bone matrix-mimetic ligands and adherent plasma membrane where numerous receptors reside. Binding is essential, but affinity does not necessarily confer function. Therefore, we did not repeat biopanning to retain as many bound peptides as possible for the following functional assays. The present study demonstrated that peptide library expressed on *E. coli* surface is capable of screening bone anabolic peptides able to act on adherent plasma membrane of osteoblasts. The proteins present in bone matrix play key roles in directing a variety of biological activities mediated by osteoblasts [5,6]. It is reasonable to conclude that ligation of those bone matrix proteins with their specific receptors expressed on osteoblast plasma membrane should elicit the cell signals that induce such biological activities. While the protein-based approach is most commonly utilized to identify the receptor or ligand for a specific protein, epitope mapping is a tedious and challenging process, even after the identification of the target molecule. In living bone matrix, osteoblasts, thought to differentiate into osteocytes, are characterized by tight cellular adhesion; as such, it is difficult to find biofunctional reagents able to bind transmembrane receptors at the adherent surface in conventional tissue cell culture systems. Herein, we reported a new approach to identify putative bone anabolic peptides that target the adherent plasma membrane of osteoblasts. This approach may also work for identification of unknown couples of ligand and receptor in the other types of adherent cells.

Conflict of interest

None.

Acknowledgments

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