Basic analysis for screening a novel bone-regenerating peptide from the phage peptide library

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ABSTRACT SUMMARY

To create a novel bone-regenerating peptide from phage peptide library, we optimized the condition to select high affinity peptides against human Tumor necrosis factor-alpha (TNF-α) and receptor activator of nuclear factor-κB ligand (RANKL). First, we constructed phage displaying peptide A and B which had affinity against TNF-α and RANKL as a template to create mutant peptide library. Both phages could not bind antigens adsorbed on a plastic plate, but they could recognize antigens captured on the streptavidin. Model selection with the mixture of peptide displaying phage and negative control phage on the antigen-coated streptavidin magnetic beads revealed that peptide displaying phage was successfully concentrated via this selection procedure. These results suggested that streptavidin-mediated antigen capturing was suitable for selecting high affinity binders from phage peptide library.

INTRODUCTION

Bone-regeneration can be an innovative therapy to overcome refractory diseases such as the periodontal disease which is accompanied with fatal bone loss. Remodeling of bone depends on the integrated activity of osteoblasts that form bone and osteoclasts that resorb bone. TNF-α and RANKL, members of the TNF superfamily, have critical roles for the bone remodeling. TNF-α and RANKL interact with TNF receptor and RANK, respectively. These interactions are required for osteoclast formation, differentiation, activation, and survival. Inhibition of these signals in bone can increase bone mass and is useful for treatment of osteoporosis. Actually, anti-human RANKL-neutralizing antibody (denosumab) has been clinically used for osteoporosis and cancer-related bone disorders. However, most of the currently available drugs inhibit osteoclastic bone resorption; drug for bone-regeneration is not established well.

Previously our group demonstrated that two peptides (peptide A and B) which had affinities against TNF-α and RANKL could promote bone-regeneration, suggesting that they can be candidates for bone-regenerating drugs2. However, affinities of these peptides are not enough; therefore it is limited to develop them as a successful drug. In this point of view, we are trying to create a novel mutant peptide which has improved affinity from wild-type peptide A and B sequence using phage display techniques. Here, we showed the optimization of the condition for selecting high affinity peptides from the phage library, before screening novel bone-regenerating peptides.

EXPERIMENTAL METHODS

Phage ELISA (direct immobilization)

Recombinant human TNF-α or human RANKL was immobilized for 12 h at room temperature (RT) on Maxisorb immunoplate (Thermo Fisher Scientific, Inc., Waltham, MA) at 5 μg/ml diluted in 50 mM carbonate bicarbonate buffer, pH9.6. Plate was blocked with PBS containing 4% skim-milk (4% MPBS) for 2 h at RT, then purified phages diluted in 2% MPBS were reacted with immobilized antigens for 2 h at RT. Bound phages were detected by anti-M13-HRP conjugate (27-9421-01; GE healthcare UK Ltd., Little Chalfont, UK) diluted in 2% MPBS and TMB substrate (Dako Denmark A/S, Glostrup, Denmark).

Biotinylation of human TNF-α and human RANKL

Recombinant human TNF-α or human RANKL were biotinylated with EZ-Link Micro Sulfo-NHS-LC-Biotinylation Kit (Thermo Fisher Scientific). Human TNF-α or human RANKL were reacted with 30 molar excess of sulfo-NHS-LC biotin in PBS (500 μg protein/ml) for 2 h on ice. Unreacted biotin was removed by desalting column. Biotinylation efficiency was calculated with Fluorescence Biotin Quantitation Kit (Thermo Fisher Scientific).

Phage ELISA (streptavidin-coated plate)

Serially diluted biotinylated human TNF-α or human RANKL was captured for 2 h at RT on Streptavidin Coated 96-Well Plates (Thermo Fisher Scientific) in PBS containing 2.5% BSA (2.5%
BSA/PBS). Plate was blocked with 2.5% BSA/PBS for 2 h at RT, then purified phages diluted in 2.5% BSA/PBS were reacted with captured biotinylated antigens for 2 h at RT. Bound phages were detected by anti-M13-HRP conjugate (GE healthcare) diluted in 2.5% BSA/PBS and TMB substrate (Dako).

Model selection with streptavidin beads

Dynabeads Streptavidin M280 (Invitrogen Corporation, Carlsbad, CA) was blocked with 2.5% BSA/PBS for 30 min at RT. Biotinylated human TNF-α or human RANKL (100 pmol each) diluted in 2.5% BSA/PBS was captured for 30 min at RT, then phage mixture (peptide A or B displaying phage : negative control phage = 1 : 9) in 4% BSA/PBS was reacted with antigen-coated beads for 30 min at RT. Beads were washed 5 times with PBS/0.1% Tween 20 and 3 times with PBS. Bound phage was eluted with 100 mM triethylamine, pH=10.5 for 5 min at RT. Eluted phage was neutralized with 1 M Tris-HCl, pH7.4. Escherichia coli TG1 strain in exponential growth phase was infected with eluted phage. Next day, single colonies were picked up and gene encoded on phagemid vector was analyzed by PCR.

RESULTS AND DISCUSSION

We performed ELISA against antigens adsorbed on Maxisorb immunoplate with peptide A and B displaying phages, but no signal was detected. The possibility was that phages could not bind antigens because adsorbed antigens were partially denatured due to physisorption on the plate surface. To select peptides against intact antigens, we chose to capture them via streptavidin.

After screening biotinylation condition of TNF-α and RANKL, we found optimized condition described in Materials and Methods (one biotin molecule was conjugated per one antigen molecule.). In phage ELISA with antigens captured on the streptavidin coated plate, dose-dependent binding were observed; whereas a negative control phage did not bind antigens (Figure 1). Next, we performed model selection on the antigen coated magnetic beads with a mixture of peptide displaying phage and negative control phage (ratio was 1:9). By analyzing insert encoded on phagemid vector in the output phage pool, peptide displaying phage clones were concentrated from 1/15 to 12/15 (peptide A; Figure 2A) and from 1/15 to 9/15 (peptide B; Figure 2B).

CONCLUSION

We optimized the selection condition to isolate high affinity binders against TNF-α and RANKL. Now we are constructing phage peptide library with billions repertories of mutant peptides to create a novel bone-regenerating peptide with higher affinity.

REFERENCES


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Figure 1. Phage ELISA against serially diluted biotinylated human TNF-α on streptavidin-coated plate. Absorbance was measured at 450 nm.

Figure 2. Insert check after model selection of phage displaying peptide A (A) or peptide B (B) against biotinylated human TNF-α. The upper bands are negative insert and the lower bands are positive insert (gene of peptide) encoded in phagemid vector.