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RANKL targeted peptides inhibit osteoclastogenesis and attenuate adjuvant induced arthritis by inhibiting NF-κB activation and down regulating inflammatory cytokines

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ABSTRACT

Peptides designed from osteoprotegerin (OPG) have previously been shown to inhibit receptor activator of NF-κB ligand (RANKL) and prevent bone loss without significantly inhibiting inflammation. The objective of this study was to develop a novel peptide with dual inhibitory activity against bone loss and inflammation using site-directed mutagenesis. Out of the three putative sites (i.e., Tyr70–Asp78, Tyr82–Glu96, and Leu113–Arg122) available on OPG for RANKL binding, Leu113–Arg122 was used as a template for peptide synthesis. Peptide mutants of the template sequence (112YLEIEFCLKHR122) were synthesized and initially screened for their inhibitory effect on RANK–RANKL binding by competitive ELISA. The most active peptide was further evaluated *in vitro* for RANKL induced osteoclastogenesis in mouse macrophage cells, and *in vivo* for Freund's complete adjuvant induced arthritis (AIA) in Lewis rats. The efficacy of the candidate peptide was compared with that of the standard drug celecoxib. The peptide YR-11 (YLEIEFSLKHR), obtained by direct substitution of cysteine with a serine residue in the template sequence, significantly ($p < 0.05$) inhibited RANK–RANKL binding, and RANKL induced TRAP activity and formation of multinucleated osteoclasts without any cytotoxicity. Administration of YR-11 peptide at the dose of 30 mg/kg (i.p.) ameliorated both bone loss and inflammation in AIA rats. To elucidate the mechanism for inhibition of inflammation in arthritic rats, serum and tissue cytokines (TNF-α, IL-1β, and IL-6) were analyzed by ELISA and RT-PCR methods. Results confirmed that YR-11 peptide inhibited pro-inflammatory cytokines in the sera and hind paw tissues of AIA rats through its suppressive effect on RANKL induced nuclear translocation of NF-κB. The results obtained in this study substantiate the therapeutic benefit of this novel peptide in the prevention of bone loss and inflammation in rheumatoid arthritis with reduced side effects.

1. Introduction

Rheumatoid arthritis is characterized by the presence of inflammatory synovitis and destruction of joint cartilage and bone. Cytokines released from synovial fibroblasts, macrophages and T-cells cause inflammation and bone erosion. Bone erosion is mainly due to differentiation of monocytes–osteoclasts [1]. The key factor for osteoclastogenesis is RANKL, a member of the tumour necrosis factor family. It is also known as osteoclasts differentiation factor (ODF), osteoprotegrin (OPG) ligand (OPGL), and TNF-related activation-induced cytokine (TRANCE). RANKL mediates osteoclastogenesis by binding to its receptor RANK on osteoclast precursor cells. The agents currently available to prevent and treat bone loss (anti-resorptives) are estrogen, bisphosphonates, and calcitonin. Since, osteoclast-mediated bone resorption contributes to bone erosions and osteopenia, inhibition of osteoclasts with antiresorptives, i.e. bisphosphonates, may be effective in preventing bone loss in inflammatory arthritis. However, in clinical trials of RA, antiresorptive therapies alone have been unable to prevent focal bone loss despite a reduction in systemic bone loss [2]. The search for treatments of bone loss has naturally included inhibitors of the RANKL cell signaling pathway. One potential inhibitor for RANKL is osteoprotegrin (OPG). Osteoprotegrin (OPG) is a 110-kDa, disulfide-linked, homodimeric glycoprotein with homology to members of the TNF receptor family, produced and released by activated osteoblast cells. OPG functions as a soluble decoy receptor for RANKL, and competes with RANK for RANKL binding and prevents

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the focal bone loss in rheumatoid arthritis [3]. The structural analysis of RANKL–OPG complex showed three OPG binding sites to RANKL: (i) OPG (Tyr70–Asp78)–RANKL (Tyr74–His86), (ii) OPG (Tyr82–Glu96)–RANKL (Ser61–Tyr68), (iii) OPG (Leu113–Arg122)–RANKL (Tyr96–Phe103). From these binding sites, Cheng et al. [4,5] developed OPG like peptidomimetics to prevent RANKL binding to RANK, and proved for the significant inhibition of bone loss in ovariectomized mice. Peptide based therapeutics are attracting now-a-days because of their high biological activity associated with low toxicity and high specificity. The additional benefits of the peptide based therapeutics include specific binding to the desired target, minimization of drug-drug interactions and less accumulation in tissues, thus reducing risks of complications due to intermediate metabolites [6]. Hence in this report, we sought to develop peptide based analogs as potential therapeutics for prevention of bone loss in rheumatoid arthritis based on the site-directed substitutions of amino acid residues by considering the third binding site of OPG-3 (112-YLEIEFCLKHR-122) with RANKL as template.

2. Materials & methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), Trypsin/EDTA, Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen Life Technologies, Inc. (Grand Island, NY, USA). XTT, sodium chloride, sodium hydroxide, and DMSO were purchased from Sigma chemical Co. (St. Louis, MO, USA). Human RANKL was procured from Promokine (Promocell, Heidelberg, Germany). Human RANK receptor was purchased from GenWay Biotech, San Diego, USA. TNF-α, IL1-β, IL-6 ELISA kits were purchased from R&D systems (Minneapolis, USA). Monoclonal antibody to human RANKL was purchased from Alexi's biochemicals, CA, USA.

2.2. Peptide synthesis and RANKL inhibition

Out of the three putative sites available on OPG for RANKL binding (i.e., Tyr70–Asp78, Tyr82–Glu96 and Leu113–Arg122), the third binding site (OPG-3) was used as a template for peptide synthesis. Candidate peptides were designed by site-directed substitutions of amino acids (YLEIEFCLKHR) present in the OPG-3 sequence (Table 1), and were synthesized at GL biochem, Shanghai, China. The purity of all the candidate peptides was >95% as determined by HPLC. The molecular weights of the peptides were characterized by NMR spectroscopy and tested for their effect on RANKL binding to the RANK receptor by ELISA method. Briefly, each peptide (10 μM) was mixed with RANKL (100 ng/ml) and incubated for 1 h at 37 °C, after which an aliquot, (100 μl) of the mixture was added to the wells (96 well plate) coated with human RANK receptor and incubated for overnight at 4 °C. Control wells contained DMSO and RANKL only. RANKL bound to RANK was detected with an anti-RANKL antibody (diluted 1:1200; 0.1 ml per well), and incubated for 2 h at 37 °C. After washing, the plate was incubated with a secondary antibody (peroxidase-conjugated goat anti-rat antibody) at 1:1500 dilutions. Wells were incubated with the chromogenic substrate TMB (Sigma), and color was measured at 450 nm. The peptides showing >90% inhibition of RANKL binding to RANK were selected for further studies.

2.3. Surface plasmon resonance analysis for RANKL inhibition

The interaction of peptides with RANKL and RANK receptor was determined by resonance equilibrium method [7] using Proteon™ XPR36 protein interaction array system (Biorad, Hercules, CA). A streptavidin coated sensor chip (proteon NLC sensor chip) was conditioned with 1 M NaCl containing 50 mM NaOH. Following conjugation of the RANK receptor (GenWay Biotech, San Diego, USA) with biotin using Lightning-Link™ Biotin Conjugation Kit (Innova Biosciences Ltd, Cambridge, UK), the biotinylated RANK (1 μg/ml in PBST) was injected over the flow cell until the response reached 600 response units (RU). RANKL (2.5 μg/ml in PBST), preincubated with YR-11 peptide at concentrations of 2.5, 5, 10, and 25 μM for 30 min at room temperature, was then injected into the system at a flow rate of 50 μl/min. The chip surface was regenerated to baseline after each cycle with 5 mM HCl. The resonance signal measured on the reference cell (containing biotin-RANK) was subtracted from the signal measured on the experimental flow cell. All experiments were performed at 25 °C. The results were fitted into 1–1 binding model using protean manger software, and the affinity constants for RANKL calculated in the presence and absence of the peptide.

2.4. Cytotoxicity study of YR-11 on isolated synovial fibroblast cells

Fibroblast cells were isolated from the synovium of the RA patient, while undergoing synovectomy at NUHS, Singapore. This study was approved by the NUHS Review Board. Fresh synovial tissues were minced and digested in a solution containing 0.1% trypsin and 0.1% collagenase [8]. Cells were used at or after the third passage at which stage they grew into a homogenous population as characterized by immunostaining for 5B5 protein. Cells were grown in DMEM supplemented with 10% FBS, and the cytotoxicity of YR-11 peptide on synovial fibroblast cells were determined by XTT assay.

2.5. In vitro osteoclastogenesis inhibition studies by TRAP assays

The mouse macrophage cell line (RAW 264.7, ATCC) was cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum and antibiotics at a density of 1×10^4 cells per well in 12-well dishes, and the cells were allowed to adhere overnight. This cell line has previously been shown to express RANK and differentiate into TRAP-positive functional osteoclasts when co-cultured with human sRANKL [9]. After replacing the medium, the cells were treated with YR-11 peptide at increasing concentrations (10, 50, and 100 μM) in duplicate wells for 1 h, and RANKL (human,

Table 1
Primers used for real time PCR.

Molecule	Forward primer	Reverse primer	NCBI reference sequence	Product size (BP)
TNF-α	AGATGTGGAACTGGCAGAGG	GAGCCCATTTGGGAACTTCT	NM_012675.3	181
IL1-β	CAGGAAGGCAGTGTCACTCA	TTTCAGCTCACATGGGTCAG	NM_031512.2	250
IL6	CCGGAGAGGAGACTTCACAG	ACAGTGCATCATCGCTGTTC	NM_012598.1	161
RANKL	GAGCGTACCTGCGGACTATC	AGGGAAGGGTTGGACACC	NM_012598.1	207
OPG	GGCTGAGTGTTCTGGTGGAC	GAGCTGCTCTGTGGTGAGGT	NM_012870.2	230
TRAP	CGCCAGAACCGTGCAGA	TCAGGCTGCTGGCTGAC	M76110	357
β Actin	GTCGTACCACTGGCATTGTG	TCTCAGCTGTGGTGGTGAAG	NM_031144.2	180