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A new vaccine targeting RANKL, prepared by incorporation of an unnatural Amino acid into RANKL, prevents OVX-induced bone loss in mice

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ABSTRACT

Bone homeostasis is maintained by a dynamic balance between osteoblastic bone formation and osteoclastic bone resorption. The receptor activator of nuclear-κB ligand (RANKL) is essential for the function of the bone-resorbing osteoclasts, and targeting RANKL has been proved highly successful in osteoporosis patients. This study aimed to design a novel vaccine targeting RANKL and evaluate its therapeutic effects in OVX-induced bone loss model. Anti-RANKL vaccine was generated by incorporating the unnatural amino acid *p*-nitrophenylalanine (pNO_2Phe) into selected sites in the murine RANKL (mRANKL) molecule. Specifically, mutation of a single tyrosine residue Tyr^{234} (Y234) or Tyr^{240} (Y240) of mRANKL to pNO_2Phe (thereafter named as $Y^{234}pNO_2Phe$ or $Y^{240}pNO_2Phe$) induced a high titer antibody response in mice, whereas no significant antibody response was observed for the wild type mRANKL (WT mRANKL). The antiserum induced by $Y^{234}pNO_2Phe$ or $Y^{240}pNO_2Phe$ could efficiently prevent osteoclastogenesis in vitro. Moreover, immunization with $Y^{234}pNO_2Phe$ or $Y^{240}pNO_2Phe$ could also prevent OVX-induced bone loss in mice, suggesting that selected pNO_2Phe-substituted mRANKL may pave the way for creating a novel vaccine to treat osteoporosis.

1. Introduction

Osteoporosis is a systemic skeletal disease characterized by a progressive loss of bone mass and microarchitectural deterioration of bone tissue, and therefore leads to increased fragility fractures [1,2]. Bone homeostasis is maintained by a dynamic balance between osteoblastic bone formation and osteoclastic bone resorption [3]. Excessive activation of osteoclastic bone resorption is a common pathological mechanism of bone loss and fractures [4,5].

Osteoclasts are multinucleated cells which are derived from macrophage/monocyte lineage hematopoietic precursors [6]. Cytokines and hormones play significant roles in Osteoclastogenesis. Particularly, macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are essential for the proliferation, survival and maturation of osteoclasts [7].

RANKL is a type II transmembrane protein found primarily on the surface of osteoblasts, activated T-cells and bone marrow stromal cells [8]. As a member of the tumor necrosis factor (TNF) cytokine family, human and mouse RANKL (hRANKL and mRANKL), which share 87% identity in protein sequence, are type II transmembrane proteins that contain a small N-terminal intracellular domain, a transmembrane region and a C-terminal extracellular domain consisting of a stalk and a receptor binding region that forms trimers [9]. RANKL exists in both membrane-bound and

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soluble forms, and both are biologically active in promoting osteoclast formation [10]. The minimal bioactive region of RANKL extends from residues 158–316 of mouse RANKL, and residues 159–317 of human RANKL. RANKL, and its receptor RANK, are a TNF superfamily receptor-ligand pair that govern the development and function of osteoclasts.

Genetic experiments have shown that targeted disruption of RANKL induced severe osteopetrosis and absence of osteoclasts in mice [9]. Previous studies also showed that an Fc-OPG fusion protein which can bind to RANKL, as well as Denosumab, a fully human monoclonal antibody to RANKL, is able to effectively inhibit bone resorption in postmenopausal women [11,12]. Despite Denosumab has been well used to treat osteoporosis in clinic, the high costs may limit its wide use. Therefore, it is necessary to explore new efficient and economical methods to treat osteoporosis.

Since RANKL is indispensable for the function of the bone-resorbing osteoclasts, and its antibody Denosumab has been well used to treat osteoporosis in clinic, engineering an effective vaccine targeting RANKL maybe a potentially therapeutic approach to osteoporosis [13–15]. In the current study, we engineered a novel vaccine targeting RANKL by introducing a *p*-nitrophenylalanine (pNO_2Phe), an unnatural amino acid derived from phenylalanine that shares high structural similarity with phenylalanine and tyrosine [16], at a single site in mRANKL. The genetic incorporation of pNO_2Phe into the self-proteins mTNF-α, retinol binding protein 4, and C5a respectively, was previously shown to elicit high titer autoantibody responses to their respective endogenous proteins [16–18]. Here, we apply this methodology to the preparation of a therapeutic vaccine, targeting RANKL, and evaluate the effects of the vaccine in mice with ovariectomy (OVX)-induced bone loss.

2. Material and methods

2.1. Mice

Female C57BL/6 mice obtained from the Animal Center of Fourth Military Medical University (Xi'an, China) were used in the experiments. All experiments were performed in accordance with the regulations approved by the Animal Care and Use Committee of the Fourth Military Medical University (Xi'an, China). All animal experiments were conducted in an age-matched, blinded, and random-grouping manner.

2.2. Construction of mRANKL expression vector, pET28a-mRANKL

The mRANKL gene was amplified from total RNA extracted from murine bone marrow by using PCR with the following primers: 5′-CCATGGGCTCATGGTTGGATGTGG and 5′- CTCGAGGTCTATGTCCTGAACTTTG. The resulting PCR product was reverse-transcribed using PrimeScript RT reagent Kit with DNA Eraser (Takara, Kyoto, Japan) and then digested with *Nco*I and *Xho*I restriction enzymes and ligated into a pET28a vector (Novagen). The recombinant vector was then modified to append an N-terminal initiation codon and C-terminal hexahistidine tags. By using the TaKaRa MutanBEST Kit (TaKaRa), site-specific incorporation of *p*-nitrophenylalanine (pNO_2Phe) into the murine RANKL gene was performed by introducing TAG amber codons at the residue intended for pNO_2Phe incorporation [16,19]. The sequences of all mRANKL constructs were verified by DNA sequence analysis.

2.3. Protein expression and purification of WT mRANKL and pNO_2Phe mRANKL

The expression of pNO_2Phe mutant protein had been previously described [16]. Briefly, The plasmids harboring the pNO_2Phe mRANKL gene were transformed into *E. coli* BL21(DE3) cells with a plasmid pEVOL-aaRS which contains a *Methanococcus jannaschii*–derived aminoacyl–tRNA synthetase/transfer RNA pair specific for pNO_2Phe [17]. The transformed strains were grown at 37 °C in the presence of 1 mM pNO_2Phe in GMML Minimal Medium culture containing 50 μg/ml kanamycin, 50 μg/ml chloramphenicol. When the OD600 reached 0.5, protein expression was then induced with 0.2% arabinose and 1 mM IPTG and incubated with shaking at 30 °C for 12–16 h. However, to express the WT mRANKL, in contrast to the pNO_2Phe mRANKL mutants, the plasmid harboring the WT mRANKL gene was transformed into *E. coli* BL21(DE3) cells and then expressed in 2 × YT medium containing 50 μg/ml kanamycin by induced with 1 mM IPTG when OD600 reached 0.5.

All purification steps were performed at room temperature. Cell pellets were sonicated in 7 M Guanidine Hydrochloride, 50 mM Tris, pH 8.0, and then the supernatant was purified by nickel-iminodiacetic acid (Ni-IDA) agarose affinity chromatography. The highly specific incorporation of the unnatural amino acid was confirmed by mass spectrometry (The Center of Mass Spectrometry, The First Affiliated Hospital of Fourth Military Medical University).

2.4. Animal immunization and detection of anti-RANKL antibodies

C57BL/6 mice (n = 6 for each group) were vaccinated subcutaneously with the WT mRANKL, pNO_2Phe mRANKL (50 μg/injection) or phosphate buffered saline (PBS) emulsified 1:1 with complete Freund's adjuvant (CFA, Sigma-Aldrich, Saint Louis MO, USA) for the initial injection (day 1) or with incomplete Freund's adjuvant (IFA) for the booster injections (day 14, 46). Sera were obtained on days 0, 14, 28, 49, 70, 91, 112, 140, and 168. The specific anti-serum titers against mRANKL were measured by enzyme linked immunosorbent assay (ELISA). Briefly, 5 mg/ml recombinant mRANKL protein (wild-type or pNO_2Phe mutants) were coated on MaxiSorp microtiter plates (Thermo Fisher Scientific Inc, Roskilde, Denmark). Serum samples were diluted 1:1000 in PBS buffer with 1% BSA and incubated in the pre-coated plates at room temperature for 2 h. After washing, bound IgG was determined with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:10,000, ProteinTech Group, Chicago, USA). The absorbance at 450 nm was recorded by a Thermo Max Microplate reader (Thermomax Technologies, Columbia, Maryland, USA).

2.5. Osteoclastogenesis in vitro

In vitro osteoclastogenesis was established as described previously [20]. Briefly, bone marrow cells (BMC) were flushed from tibias and femurs of 6–8 week-old female C57BL/6 mice, cultured in alpha-MEM containing 10% FBS and 25 ng/ml M-CSF after treating with RBC lysis buffer (eBioscience). After 1 day incubation, non-adherent cells were removed and cultured in 48-well plates in alpha-MEM supplemented with 10% FBS and 50 ng/ml M-CSF for 2 days. Newly adherent cells were used as bone marrow macrophages (BMMs). Subsequently, Osteoclast differentiation was induced by addition of 50 ng/ml M-CSF and 100 ng/ml RANKL and in the present or absent of 3% immunogen-induced antiserum for a further 6 days. The multinucleated cells were stained for tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich Co, Cat No.387A) and more than 3 nuclei TRAP-positive cells were identified as Osteoclasts.

2.6. Ovariectomy and bone loss preventive vaccination

12-week-old C57BL/6 female mice (n = 6 for each group) were randomly subjected to either sham operation or bilateral ovariectomy under anesthesia with intraperitoneal pentobarbital sodium (100 mg/kg BW). The OVX mice were randomly assigned into four

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