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Inhibition of osteoporosis by the αvβ3 integrin antagonist of rhodostomin variants

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ABSTRACT

Integrins are heterodimeric cell surface receptors that mediate cell–cell and cell–matrix interaction. The vitronectin and osteopontin receptor αvβ3 integrin has increased expression levels and is implicated in the adhesion, activation, and migration of osteoclasts on the bone surface as well as osteoclast polarization. αvβ3 integrin plays an important role in osteoclast differentiation and resorption. In addition, Arg-Gly-Asp (RGD)-containing peptides, small molecular inhibitors, and antibodies to αvβ3 integrin have been shown to inhibit bone resorption in vitro and in vivo. Here we examined the effects of a disintegrin HSA-ARLDDL a genetically modified mutant of rhodostomin conjugated with human serum albumin, which is highly selective of αvβ3, on RANKL-induced osteoclastogenesis and ovariectomy (OVX)-induced osteoporosis. In RANKL-induced osteoclastogenesis, HSA-ARLDDL significantly inhibited osteoclast formation, and IC_{50} was at nM range. Post-treatment HSA-ARLDDL also inhibits osteoclast formation. Furthermore, weekly administration of HSA-ARLDDL significantly inhibits the increase in serum bone resorption marker levels and decrease in cancellous bone loss in tibia and femur induced by OVX. On the other hand, HSA-ARLDDL did not affect the differentiation and calcium deposition of osteoblasts. These results indicate that the highly selective and long-acting αvβ3 integrin antagonists could be developed as effective drugs for postmenopausal osteoporosis.

1. Introduction

The adult skeleton undergoes a remodeling process that involves osteoblast-mediated synthesis and osteoclast-mediated resorption of the bone. However, osteoporosis, which is caused by the imbalance of bone remodeling, can be characterized by low bone mass associated with an increased risk of fracture (Kanis, 1997). The imbalance of remodeling can result in defects in the skeletal structure and function. When bone resorption exceeds bone synthesis because of an increase in osteoclast differentiation or activity, it results in progressive bone loss, such as postmenopausal osteoporosis, periodontal disease, and rheumatoid arthritis (Rodan and Martin, 2000). Osteoclast-mediated bone resorption includes multiple steps: differentiation of osteoclast progenitors to mononuclear prefusion osteoclasts, fusion of precursor osteoclasts to multinucleated osteoclasts, and attachment to bone surface (Boyle et al., 2003). However, integrins, particularly αvβ3, are key mediator of osteoclast differentiation and bone resorption (Nakamura et al., 2007).

Integrins, a superfamily of cell surface receptors that mediate cell–cell and cell–matrix interactions, are heterodimeric receptors composed of α and β subunits. It has been reported that, among various integrins, osteoclasts express very high levels of αvβ3 integrin as vitronectin and osteopontin receptors, and it is well established that αvβ3 integrin is a central molecule for osteoclast-mediated bone resorption (Nakamura et al., 1998; Nakamura, 2007). Chambers et al. (1986) first indicated that αvβ3 integrin plays an important role in osteoclast function using a monoclonal antibody 13C2 to inhibit osteoclast-mediated bone resorption in vitro, and Davies et al. (1989) further identified the antigen of this antibody as αvβ3 integrin, which was subsequently demonstrated to be the most abundant integrin in osteoclasts (Clover et al., 1992). In addition, β3−/− mice have dysfunctional osteoclasts and aged β3−/− mice become progressively osteosclerotic (McHugh et al., 2000). It has also been reported that β3−/− mice are protected from ovariectomy (OVX)-induced bone loss

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(Zhao et al., 2005). Pharmacological blockade of integrin using integrin antagonists, including functional blocking antibodies, ligand-containing disintegrins, and small molecule antagonists has been reported to inhibit osteoclast differentiation and bone resorption in vitro and in vivo (Horton et al., 1991; Fisher et al., 1993; Engleman et al., 1997; Murphy et al., 2005).

Here we examined the antiosteoporosis function of a selective αvβ3 antagonist, a protein derived from the mutant of the RGD domain of rhodostomin. The disintegrin has been conjugated with human serum albumin to prolong the protein half-life and reduce immunogenicity. We found a potential candidate for the treatment of bone diseases, such as OVX-induced osteoporosis or Paget's disease.

2. Materials and methods

2.1. Animals

All protocols complied with institutional guidelines and were approved by Animal Care Committees of Medical College, National Taiwan University. Eight to ten weeks-old male Sprague-Dawley rats (250–300 g) were purchased from BioLasco (Taipei, Taiwan) and 2 months-old female CD-1/ICR mice (Imprinting Control Region) (22–28 g) were purchased from Laboratory Animal Center of Medical College, National Taiwan University.

2.2. Materials

Alendronate, alizarin red-S, L-ascorbic acid, cetylpyridinium chloride, β-glycerophosphate and tartrate resistance acid phosphatase (TRAP) staining kit were purchased from Sigma Chemical Company (St. Louis, MO, USA). Recombinant mouse receptor for activation of nuclear factor kappa B ligand (RANKL) and mouse M-CSF were from R & D system (Minneapolis, MN, USA). C-terminal telopeptides of type-I collagen kit (Rat-Laps) was purchased from Immunodiagonstic Systems Ltd (Boldon Colliery, Tyne & Wear, UK). The derivative of rhodostomin, HSA (C34S) (human serum albumin (C34S) conjugated)-ARLDDL (abbreviated as HSA-ARLDDL), and rabbit antibody against rhodostomin were provided by Professor Chuang (Cheng-Kung University).

2.3. Expression of HSA (C34S)-ARLDDL in P. pastoris and purification

The structural gene of HSA (C34S) was constructed using HSA (invitrogen: clone ID: IOH23065) as a template, and the mutation of C34S was produced by two-step polymerase chain reaction (PCR). The first PCR was amplified with the sense primer containing C34S mutation site and with the antisense primer containing Kpn I, Sac II restriction site and a TTA stop codon. The second PCR was amplified with the sense primer containing BstB I restriction site and signal sequence and with the antisense primer containing Kpn I, Sac II restriction site and a TTA stop codon. The secretion signal sequences of HSA prepro peptide, the α factor prepro peptide from Saccharomyces cerevisiae, or preHSA and pro α factor peptide was used for secretory protein expression. The structural gene of ARLDDL was amplified by PCR with the sense primer containing Kpn I restriction site and the spacer region containing GS sequence and with the antisense primer containing Sac II restriction site and a TTA stop codon. The PCR products of HSA (C34S) with the secretion signal peptide and Rho ARLDDL mutant with the spacer region were digested using Kpn I restriction enzyme and then were ligated. The resulting gene product was cloned into the BstB I and Sac II sites of the yeast recombination vector. The recombinant plasmid was then transformed into an Escherichia coli XL1-blue strain, and the colonies were selected using the agar plates with low salt LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar at pH 7.0) and 25 μg/ml antibiotic Zeocin. The E. coli XL1-blue colonies were picked, and the plasmid DNA was isolated. After the clone was confirmed by sequencing the insert, a total of 10 μg plasmids were digested with Sac I to linearize the plasmids. Pichia host strain was transformed with the linearized constructs by a heat shock method, using a Pichia EasyComp™ kit from Invitrogen® or electroporation. The transformant integrated at the 5′ AOX1 locus by a single crossover. PCR was used to analyze Pichia integrants to determine if the HSA (C34S)-ARLDDL gene has been integrated into the Pichia genome. The colonies were selected on agar plates containing YPD (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and 100 μg/ml Zeocin. A number of clones with multiple copies of HSA (C34S)-ARLDDL gene insertions were selected to pick the clone with the highest protein expression. The resulting recombinant HSA (C34S)-ARLDDL contained 585 amino acids of HSA, a spacer containing 17 amino acid residues, and 68 amino acids of rhodostomin ARLDDL mutant.

HSA (C34S)-ARLDDL was produced by following protocols previously described (Guo et al., 2001). The proteins were produced as follows: a 100 μl of cells stock grew at 30 ℃ in 100 ml of yeast nitrogen base (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose) containing with 100 μg/ml Zeocin for 48 h. Cells were then transferred into 900 ml of YPD medium. After another 48 h, the cells were collected by centrifugation and grown in 1 l of minimal methanol medium (1.34% yeast nitrogen base (YNB)) with ammonium sulfate without amino acids and 4×10–5% biotin. A total of 1% methanol was added once every 24 h to induce protein expression for 2 days. The supernatant was collected by centrifugation and dialyzed twice against 10 l of H_2O and once against 5 l of binding buffer (50 mM phosphate buffer at pH 8.0). The dialyzed solution was loaded into the Blue sepharose column and proteins were eluted by elution buffer containing 50 mM $NaHCO_3$ and 1.5 M NaCl at pH 10.5. Proteins were further purified by C18 reversed-phase HPLC with a gradient of 20–50% acetonitrile. The purification of recombinant Proteins was greater than 95% pure as judged by SDS-PAGE.

2.4. RANKL-induced osteoclastogenesis

Osteoclastogenesis was examined in the presence of RANKL and M-CSF by using bone marrow cells as precursors (Lin et al., 2008). Bone marrow cells were prepared from tibiae and femurs of male Sprague-Dawley rats by flushing the bone marrow cavity with α-MEM. The non-adherent cells (hematopoietic cells) were collected 24 h later and used as osteoclast precursors. Cells were seeded at 10^6 cells/well (0.5 ml) in the presence of testing substances and human recombinant soluble RANKL (50 ng/ml) and murine M-CSF (20 ng/ml) for 6 days and culture medium was changed every 3 days. Osteoclast formation was confirmed by TRAP staining. In brief, after 6 days' culture, the cells were washed twice with PBS and then fixed in PBS containing 4% paraformaldehyde for 2 min. Cells were treated with TRAP staining kit (70 μg/ml Fast Garnet GBC base solution, 125 μg/ml Naphthol AS-Bl phosphoric acid, 100 mM acetate, and 6.7 mM tartrate) at 37 °C for 1 h in the dark. Cells were then carefully washed with distilled water and air dried for photography and counting. The characterization of osteoclast is TRAP-positive with more than 3 nuclei.

2.5. Primary osteoblast cultures

Primary osteoblastic cells were obtained from the calvaria of 1-day-old Sprague-Dawley rats (Lin et al., 2008). In brief, the calvaria of fetal rats were dissected with aseptic technique. The soft tissues were removed under dissecting microscope. The calvaria were divided into small pieces and were treated with 1 mg/ml collagenase solution for 20–30 min at 37 °C. The next two 20 min sequential collagenase digestions were then pooled and filtered through 70 μm nylon filters (Falcon, BD Biosciences, San Jose, CA, USA). The cells were grown on the plastic cell culture dishes in 95% air-5% CO_2 with α-MEM, which