Bone 49 (2011) 623–635

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

The effects of the cathepsin K inhibitor odanacatib on osteoclastic bone resorption and vesicular trafficking

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ARTICLE INFO

Article history:
Received 1 March 2011
Revised 10 June 2011
Accepted 14 June 2011
Available online 22 June 2011

Edited by R. Baron

Keywords:
Cathepsin K inhibitor
Osteoclast
Bone resorption
Transcytosis
Vesicular trafficking
Osteoporosis

ABSTRACT

Odanacatib (ODN) is a selective, potent and reversible inhibitor of cathepsin K (CatK) that inhibits bone loss in postmenopausal osteoporosis. Evidence from osteoclast (OC) formation from bone marrow of CatK$^{-/-}$ mice or human OC progenitors treated with ODN, demonstrated that CatK inhibition has no effect on osteoclastogenesis or survival of OCs. Although having no impact on OC activation, ODN reduces resorption activity as measured by CTx release (IC_{50} = 9.4 nM) or resorption area (IC_{50} = 6.5 nM). While untreated cells generate deep trail-like resorption lacunae, treated OCs form small discrete shallow pits. ODN leads to significant accumulation of intracellular vesicles intensely stained for CatK and TRAP. CatK (+) vesicles localize toward the basolateral and functional secretory membranes of the polarized OC and TRAP(+) vesicles evenly distribute in the cytoplasm, suggesting that ODN disrupts multiple vesicular trafficking pathways. Intracellular levels of both precursor and mature TRAP were increased by 2-fold and the pre-pro and mature CatK by 6- and 2-fold in ODN-treated OCs compared to untreated controls. ODN treated OC accumulates labeled degraded bone matrix proteins in CatK containing vesicles. In summary, ODN treatment inhibits bone resorption by blocking degradation of demineralized collagen in the resorption lacunae, and retarding transcytosis for further processing of degraded proteins.

Introduction

Osteoclasts (OCs) are large multinucleated cells derived from hematopoietic origin. During bone resorption, activated osteoclasts are morphologically characterized by at least four different membrane domains: the sealing zone, ruffled border, the functional secretory domain and the basolateral membrane [1]. During formation of the sealing zone the OC establishes an isolated compartment, the resorption lacunae, where resorption occurs. The sealing zone is organized by cytoskeletal proteins with the integrin $\alpha_v\beta_3$ mediating the initial interaction between the cellular membrane and the bone extracellular matrix [2]. Transport and fusion of vacuolar H^+-ATPase and lysosomal acidic vacuoles to the OC ruffled border membrane maintain the low pH in the resorption lacunae. These are necessary for the dissolution of the mineral component as well as for the establishment of an optimal environment for lysosomal protease-mediated degradation of the demineralized bone proteins [3].

Cathepsin K (CatK) is a lysosomal cysteine protease, abundantly expressed in OC, that can degrade protein components of the demineralized bone matrix, most notably type-1 collagen [4]. The physiological role of CatK has been validated in pycnodysostosis, an autosomal recessive osteosclerotic skeletal dysplasia characterized by high bone mineral density (BMD), acroosteolysis of the distal phalanges, short stature and skull deformities, associated with loss-of-function mutations in the CatK gene [5,6]. Electron microscopy of OC from pycnodysostosic patients revealed cytoplasmic vesicles containing partially digested collagen fibrils [7]. Mice with CatK targeted disruption have a phenotype similar to pycnodysostosis in humans. Adult CatK$^{-/-}$ mice show higher BMD via reduction of bone resorption, and significantly increased bone formation rate in the central and distal femur, leading to thicker cortices as compared to wild-type mice [7–9]. This collective evidence supports the role of CatK in osteoclastic bone resorption and suggests that inhibition of this protease represents a potential therapeutic approach to treat human disease associated with increased OC-mediated bone resorption, including postmenopausal osteoporosis or metastatic bone disease.

OCs secrete metalloproteinases and cathepsins into the resorption lacunae which degrade collagen, however the levels of CatK far exceeds other collagenases confirming its prominent role in bone degradation [7,10]. Furthermore, the property of CatK is unique among the mammalian proteases, due to its ability to cleave both the telopeptides and helical region of collagen type I [11]. Dissolution of bone matrix proteins results in various degradation products including collagen fragments, proteoglycans and growth factors, accumulating in the resorption pit. Since the sealing zone is impermeable to molecules larger than 10 kDa, the proteinaceous degradation products of resorption are endocytosed by the OC at the ruffled border and transported to the functional secretory domain by

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doi:10.1016/j.bone.2011.06.014

transcytotic vesicles [12]. The localization of CatK in intracellular vesicles along with collagen fibrils provides evidence that further degradation of the collagen products occurs intracellularly [13].

Odanacatib (ODN, MK-0822) is a highly potent inhibitor of human CatK ($IC_{50} = 0.2$ nM) and displays ~300-fold selectivity against cathepsin S and ≥1000-fold selectivity against all other cathepsins [14]. Enzyme kinetic studies demonstrated that ODN behaves as a reversible time-dependent inhibitor of human CatK. ODN was previously reported to inhibit OC-mediated bone resorption *in vitro* [14]. In OVX rabbits, ODN dose-dependently increased bone mineral density and improved bone strength [15]. Furthermore, ODN does not reduce OC number on bone surfaces and has no effect on the bone formation rate in this model [15]. In a phase II clinical trial, once weekly ODN treatment dose-dependently increased lumbar spine and total-hip BMD and suppressed bone turnover markers in postmenopausal women with low BMD [16].

Previous studies described the cellular effects of several non-selective cysteine protease inhibitors on osteoclast activities [17,29]. In this study, we re-confirmed previous findings on the selective and abundant expression of CatK in OCs from murine and human bones. Using OC formation assays from bone-marrow isolated from $CatK^{-/-}$ mice in the presence of RANKL and M-CSF, we also characterized the effects of CatK inhibition on osteoclastogenesis, function, and survival *in vitro*. More importantly, our study demonstrated the use of the highly selective CatK inhibitor ODN, results in reduction of OC resorption activity, yet does not affect generation or survival of the multinucleated tartrate-resistant acid phosphatase (TRAP) positive OCs. We have also provided additional mechanistic insight on how ODN inhibition of CatK produces an overall reduction in bone resorption efficiency via interrupting intracellular vesicular trafficking.

Materials and methods

Osteoclast cultures

Murine bone marrow derived osteoclasts

Whole bone marrow from the long bones were isolated from 4 to 6 week old $CatK^{-/-}$ and wild type mice as previously described [18]. One million cells were plated per bovine cortical bone slices in α-MEM, 10% FBS, 30 ng/ml of soluble RANKL (R&D, Minneapolis, MN) and 10 ng/ml of M-CSF (Invitrogen, Camarillo, CA). Media was replaced with 50% volume with and without ODN every 2 days, and culture was terminated on day 13.

Human osteoclasts

Human osteoclast precursors at $\sim 3 \times 10^4$ cells/cm^2 (Poietics®, Lonza, Walkersville, MD, USA) were cultured on dentine or bone slices ($n = 3$–6 per condition) in differentiation media with M-CSF and RANKL according the manufacturer's protocol. To prepare for ALN treatment, bone slices were preincubated in media containing 3 μM ALN, ODN was added 2 days after cell seeding on bone slices. Media with and without drug were refreshed every 2 days, and the culture was terminated on day 7. Aliquots from media were collected for measuring CTx (CrossLaps®, IDS, Fountain Hills, AZ, USA). Bone slices were fixed in 4% paraformaldehyde, stained for tartrate-resistant acid phosphatase (TRAP) for analysis of osteoclast number, and processed for measuring resorption area or immunohistochemical staining.

To synchronize mature human OC, precursors were seeded on petri dishes at 2.5×10^3 cells/cm^2 in differentiation media for 8–9 days and harvested with 0.05% Trypsin/EDTA. To assess cell survival, differentiated OC at $\sim 7 \times 10^4$ cells/cm^2 were re-seeded on bovine bone slices with or without 100 nM ODN. Bone slices were fixed on days 2, 4, 6, and 12 with no media changes. Samples were stained for TRAP activity, and OC number.

Western blots

Mature differentiated OCs plated on bone slices were treated with vehicle or 100 nM ODN for 4 days. Protein lysates were collected in RIPA buffer (Sigma) supplemented with protease inhibitors (Complete; Roche, Mannheim, Germany), 1 mM PMSF, 5 μg/ml leupeptin and 1 μM E64d (Roche Biochemicals). The lysates were centrifuged, separated on 4–12% SDS-PAGE electroblotted onto Hybond-P PVDF membrane (GE Life Sci., Piscataway, NJ, USA), after blocking with 5% non-fat dry milk (w/v) in TBST. The membrane was incubated with either anti-human CatK mAb (Calbiochem, USA) or anti-human TRAcP mAb (Invitrogen, Carlsbad, CA, USA) with 3% non-fat dry milk in TBST for 2 h, followed with appropriate HRP-conjugated IgG as secondary antibodies (Invitrogen, Carlsbad, CA, USA). The signals were detected using enhanced chemiluminescence and exposed to film (ECL Western Detection Reagents, and Hyperfilm, GE Life Sci., Piscataway, NJ, USA).

Microscopy

Localization of CatK with transmission electron microscopy

Immunolabeling for CatK was performed in thin sections of mouse femoral primary spongiosa. Samples were prepared as previously described [19]. Briefly, grids containing bone sections were incubated overnight at 4 °C with chicken anti-human CatK pAb (IDS Inc., Fountain Hills, AZ, USA) diluted 1:25 and washed in TBS. Grids were then incubated in rabbit anti-chicken IgG conjugated to 10 nm gold for transmission electron microscopy (BioCell, Goldmark Biol., Phillipsburg, NJ) for 1 h at room temperature. Sections were stained with uranyl acetate and photographed in a Phillips CM 12 electron microscope (Mahwah, NJ). Negative controls included sections which were incubated with nonspecific rabbit serum instead of primary antibody or with secondary antibodies alone. The labeling profile observed in negative controls was considered to be non-specific.

Visualization of resorption pits with scanning electron microscopy

Resorbing OCs on bone slices were washed gently in PBS and fixed for 10 min in 2.5% glutaraldehyde/paraformaldehyde in 0.1 M sodium cacodylate. The bone slices were rinsed and stored in water. Critical point drying for sample preparation, osmium coating, and visualization with scanning electron microscopy was performed by Structure Probe, Inc (West Chester, PA, USA).

Confocal microscopy

Sample preparation was as previously described [20]. Immunostaining samples were viewed under Nikon Eclipse confocal microscopy TE 2000 at 60× oil objective. Serial z-scanning images were taken by Perkin Elmer ultraview camera and Velocity software (Perkin Elmer, Waltham, MA).

Immunostaining and immunohistochemistry

Immunostaining

Differentiated OCs were cultured on cortical bone slices for 4 days, fixed with 4% paraformaldehyde and permeabilized in 1% BSA and 1% Triton-X-100. Samples were blocked with 1% BSA and stained with various primary antibodies: rabbit anti-bovine collagen-1 antibody (Millipore, Billerica, MA); anti-paxillin and anti-vinculin mAb, as previously described [20]. For double labeling with CatK and TRAP, the cells were stained first with mouse anti-human ACP5 pAb (Abnova, Taipei City, Taiwan), and followed with the appropriate secondary antibody. Unlabeled antibody was saturated by incubation with unconjugated goat anti-mouse IgG Fab (Jackson ImmunoRes., West Grove, PA, USA). Then they were stained with mouse anti-human CatK mAb (Calbiochem, City State) and followed with the appropriate secondary antibody. F-actin was visualized with 5 U/ml of phalloidin