Calcified Tissue International
https://doi.org/10.1007/s00223-018-0472-7

ORIGINAL RESEARCH

A Mild Inhibition of Cathepsin K Paradoxically Stimulates the Resorptive Activity of Osteoclasts in Culture

Dinisha Cyril Pirapaharan[1] · Kent Søe[1] · Preety Panwar[2] · Jonna Skov Madsen[3] · Marianne Lerbæk Bergmann[3] · Martin Overgaard[4] · Dieter Brömme[2] · Jean-Marie Delaisse[1]

Received: 13 July 2018 / Accepted: 30 August 2018

Abstract

Cathepsin K (CatK) inhibition allows reducing bone resorption with specific advantages compared to the existing anti-osteoporosis drugs. Its clinical use appears even more promising with the recent development of ectosteric inhibitors. A confusing observation, however, is that a low dose of the active site CatK inhibitor odanacatib (ODN) was reported to decrease bone mineral density and increase serum levels of the bone resorption marker carboxy-terminal collagen crosslinks (CTX). The present study provides a possible explanation for this paradox. The resorptive activity of human osteoclasts seeded on bone slices was inhibited when subjected to ODN at doses of 20 nM, but about 100-fold lower doses induced a significant increase in CTX levels and in eroded surface (12 repeats). This low-dose-induced stimulation was prevented by inhibition of non-CatK cysteine proteinases, thereby indicating that the stimulation results from an interplay between CatK and other cysteine proteinases. Effective interplay between these proteinases was also shown in enzymatic assays where the CatK-mediated degradation of collagen was enhanced upon addition of cathepsins B or L. Furthermore, extracts of osteoclasts subjected to a low dose of ODN showed higher levels of cathepsin B compared with extracts of control osteoclasts. In conclusion, the low-dose-induced stimulation of resorption observed in the clinical study can be reproduced in osteoclasts cultured in the absence of any other cell. Our data support an osteoclast-intrinsic mechanism where a mild inhibition of CatK results in increased levels of other proteinases contributing to the collagen degradation process.

Keywords Bone resorption · Cathepsin K · Osteoporosis · Odanacatib · Osteoclast

Introduction

Cathepsin K (CatK) is the proteinase required for collagen degradation by osteoclasts during bone resorption [1–3]. CatK inhibition recently became recognized as an attractive strategy to reduce bone resorption in situations like osteoporosis: it was shown to prevent bone loss, to reduce fracture risk, and its anti-resorptive activity does not appear to reduce bone formation as much as do the current anti-osteoclastic drugs used in the clinic [4–7]. Thus, CatK inhibition offers specific advantages as anti-osteoporotic treatment. Promise for its effective clinical use became even greater with the recent development of potent ectosteric CatK inhibitors, which appear devoid of the side effects shown by the earlier active site-directed inhibitors [8, 9].

However, studying the influence of CatK inhibition on bone has also revealed a confusing effect: a clinical phase 2 trial testing increasing doses of odanacatib (ODN) showed that the higher doses decreased serum levels of the resorption marker

✉ Kent Søe
kent.soee@rsyd.dk

✉ Jean-Marie Delaisse
Jean-Marie.Delaisse@rsyd.dk

[1] Department of Clinical Cell Biology, Vejle Hospital/Lillebaelt Hospital, Institute of Regional Health Research, University of Southern Denmark, 7100 Vejle, Denmark

[2] Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of British Columbia, Vancouver, Canada

[3] Department of Biochemistry and Immunology, Vejle Hospital/Lillebaelt Hospital, Institute of Regional Health Research, University of Southern Denmark, 7100 Vejle, Denmark

[4] Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Odense, Denmark

Published online: 07 September 2018

carboxy-terminal collagen crosslinks (CTX) and increased bone mineral density (BMD) as expected, but paradoxically, the lowest dose led to increased levels of CTX and decreased BMD compared to the placebo control group [10]. This observation deserves attention not only because of its scientific interest, but also because of its clinical implications, since low drug compliance is anticipated to have the same effect as the low-dose treatment [10]. Therefore, we herein address the mechanism whereby a mild inhibition of CatK stimulates bone resorption.

Silencing CatK activity is known to induce compensatory regulations of cytokines and proteinases in both osteoclasts and osteoblast lineage cells, as investigated in the context of pycnodysostosis and preclinical bone resorption models in mice [11–14]. This type of compensation for lack of cysteine proteinase activities has also been reported in other tissues [15–17]. We speculate that the low-dose effect is likely to involve a similar type of mechanism and hypothesize the following scenario. A low dose of ODN lets a large proportion of CatK molecules uninhibited, but may be enough to induce "compensatory" proteinases, such as cathepsin B (CatB), cathepsin L (CatL), and matrix metalloproteinases (MMPs). Such proteinases are inefficient to degrade collagen fibers by themselves mainly because they lack CatK's powerful triple-helix-cleavage activity [18, 19]. However, they degrade the proteoglycan bridges involved in the assembly of the collagen fibrils [18], exert telopeptidase activity, and act as gelatinases on unwound triple helices once they are cleaved [1, 19], and the osteoclastic MT1-MMP [20] may even cleave native triple helices at a single point. One may thus expect that these proteinases can exert a helping function, and test tube experiments showed that their addition to triple-helix-cleaving proteinases actually leads to accelerated collagen degradation [21–23]. In the present study, we test whether this scenario might explain the stimulation of resorption induced by a low dose of ODN. Furthermore, we test whether the enzymatic machinery of the osteoclast alone is enough to respond to a low dose of ODN without requiring complex interactions between different cell types.

Therefore, we first investigated the effect of increasing doses of ODN on the resorptive activity of osteoclasts cultured in the presence or absence of inhibitors of putative helper proteinases. Second, we investigated whether collagen degradation achieved by CatK in enzymatic assays is accelerated in the presence of the putative helper proteinases. Finally, we search for indications of enhancement in possible helper proteinases in response to the low dose.

Materials and Methods

Osteoclast Generation In Vitro

Primary human osteoclasts were generated from $CD14^+$ cells isolated from peripheral blood, as previously described [24] (local ethical committee, 2007–0019; written consent obtained from each donor). In brief, $CD14^+$ cells were isolated using BD IMag^{TM}Anti-Human CD14 Magnetic Particles-DM (BD Biosciences, San Jose, CA) after Ficoll-Plaque (Amersham, GE Healthcare, Little Chalfront, UK) purification. Cells were seeded at a density of 5×10^6 cells/T75 culture flasks (Greiner, Frickenhauser, Germany) in αMEM (Invitrogen, Taastrup, Denmark) containing 10% FBS (Sigma-Aldrich, St. Louis, Mo, USA or Biological Industries, Kibbutz Beit-Heamek, Israel), and 25 ng/mL human macrophage colony-stimulating factor (M-CSF) (R&D System, Abingdon, UK) and cultured at 37 °C in a 5% CO_2 atmosphere for 2 days [25]. The cells were differentiated into mature osteoclasts through the addition of 25 ng/mL nuclear factor kappa-B ligand (RANKL) and 25 ng/mL M-CSF (R&D System, Minneapolis, MN). The cells were cultured for 7 days with medium change twice.

Inhibitors

ODN ((2S)-*N*-(1-cyanocyclopropyl)-4-fluoro-4-methyl-2-[[(1S)-2,2,2-trifluoro-1-[4-(4-methylsulfonylphenyl)phenyl]ethyl]amino]pentanamide) (Selleckchem.com, Houston, TX, USA) [10] and L873724 ((2S)-*N*-(cyanomethyl)-4-methyl-2-[[(1S)-2,2,2-trifluoro-1-[4-(4-methylsulfonylphenyl)phenyl]ethyl]amino]pentanamide) (a generous gift from Merck, Rahway, NJ, USA) [26] were used as specific inhibitors of CatK (IC_{50} (nM) of ODN for CatK: 0.2, CatB: 1034, CatL: 2995, and CatS: 60; IC_{50} (nM) of L873724 for CatK: 0.2, CatB: 5239, CatL: 264, and CatS: 178). Both were solubilized in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). K11097 (Khepri Pharmaceuticals, Inc) [27] is a potent inhibitor of CatL, CatS, and CatB with second-order rate constants of 9,200,000 M^{-1} s^{-1}, 56,000,000 M^{-1} s^{-1} and 420,000 M^{-1} s^{-1}, respectively, but is a weak inhibitor for CatK with second-order rate less than 300 M^{-1} s^{-1} [27]. It was solubilized in DMSO. K11017 [27] (Khepri Pharmaceuticals, Inc) and E64 (*trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane) (Sigma-Aldrich, Copenhagen, Denmark) are pan cysteine proteinase inhibitors and were solubilized in DMSO. GM6001, *N*-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (Merck life Science, Hellerup,

Denmark) is a broad inhibitor of MMPs solubilized in DMSO. The same amount of DMSO was added to the controls, which resulted in a final concentration of 0.1% DMSO in each condition.

Bone Resorption Assay

Mature osteoclasts (100 000 cells/96-well) were detached with accutase (PAA, Pasching, Austria) and re-seeded on bovine cortical bone slices with a thickness of 0.4 mm (BoneSlices.com, Jelling, Denmark) for 72 h in the presence of 25 ng/mL M-CSF and 25 ng/mL RANKL [24], and in the presence or absence of various proteinase inhibitors. For each condition, we used four to five bone slices, as indicated in the figure legends. Resorption cavities were stained with toluidine blue [28] (Sigma-Aldrich). The total eroded surface was assessed by using a 100-point grid (Pyser-SGI, Edenbridge, UK) placed in the ocular of an Olympus BX50 microscope (Olympus, Ballerup, Denmark) as previously reported [25]. In brief, the total eroded surface is expressed as percentage of the total bone surface. The eroded surface was subdivided into pit and trench surfaces. As previously described [25], pits are defined as round excavation with well-defined edges and the ratio between the length and the width did not exceed two. Trenches were defined as long elongated and continuous excavations with well-defined edges and were at least two times longer than its width. The prevalence of trenches is also expressed as percentage per total eroded surface. The total number and maximum demineralization depths of pits and trenches were determined as described previously [28]. The levels of CTX in the conditioned medium were measured using semi-automated Cobas E602 robot by using β-CrossLaps (Roche Diagnostic, Denmark). All bone slices from a single experiment were placed in a random order by another person prior to quantification. Thus, the observer was blinded during all quantifications.

Detection of Proteinase Levels at "the Low ODN Dose" by Western Blotting

Mature osteoclasts were cultured as explained above in the presence of increasing doses of ODN, in order to identify the doses that induce increased resorption. In parallel, 24-h cultures with osteoclasts from the same donor were performed on ten bone slices per condition, in order to make osteoclast lysates. The osteoclasts cultured in the same condition were lysed as a pool in same lysis buffer and stored at − 20 °C. Equal amount of protein was loaded on Criterion precast 10% Bis–Tris gels and was run according to description by the suppliers (Bio-Rad, Hercules, CA, USA). Protein from the gel was blotted onto a nitrocellulose membrane (Bio-Rad) or PVDF membrane (Bio-Rad) and was blocked with TBS containing 0.1% Tween 20 and 3% (w/v) BSA for 1 h. Thereafter, it was incubated with primary mouse-αhCatB monoclonal IgG2a antibody (clone CA10, epitope within heavy chain of mature CatB; Abcam, Cambridge, UK) or primary mouse-αhCatL monoclonal IgG1 antibody (clone 33/2, epitope within amino acid residues GYGFEST (169–175) in mature CatL; Abcam, Cambridge, UK) in TBS + 3% BSA at 4 °C overnight. The membrane was washed and incubated with secondary HRP-coupled goat-antibody [anti-mouse antibody (Amersham ECL WB system, GE Health Care)] in TBS + 3% BSA for 1 h at room temperature. The protein bands were detected using ChemiDoc Imaging System (Bio-Rad). The membrane was stripped with stripping buffer (0.1 M glycine, 20 nM magnesium acetate and 50 mM potassium chloride, pH 2.2) for 2 × 10 min at room temperature and incubated with monoclonal mouse-αhβActin (Sigma-Aldrich, St. Louis, MO, USA) used as control. All incubation and washing steps were done using a rocking table. Results were analyzed using Image Lab software and the molecular weights were determined using MW analysis tools (Bio-Rad).

Test-Tube Collagenolysis Assays

Insoluble type I collagen fiber degradation was performed as described in [8]. 1 mg collagen fibers isolated from mouse tails was incubated in the presence and absence of 1 µM CatK, either alone or in the presence of 200 nM CatB and 200 nM CatL in 100 mM sodium acetate buffer pH 5.5, containing 2.5 mM DTT and EDTA for 6 h at 28 °C. Cathepsins were expressed in Pichia pastoris and purified as previously described [29]. The reaction was stopped with E64 (Sigma). Collagen fibers were then processed for SEM, while the reaction supernatants were processed for hydroxyproline measurement.

Data Analyses and Statistical Analyses

All graphs and statistical analyses were performed using GraphPad Prism software, version 6 (GraphPad Software, LA Jolla, Ca, USA). Data were tested for normality by using D'Agostino & Pearson omnibus normality test, and this allowed us to decide whether the statistical test should be parametric or non-parametric. The results of statistical analyses for datasets shown in figures were based on one-way ANOVA Kruskal–Wallis test and Dunn's multiple comparisons test (Figs. 1, 3), Mann Whitney test (Fig. 4b), Spearman's test (Fig. 2a), and paired *t* test (Fig. 2b, c). All *p* values were based on two-tailed analyses *p* values and the level of significance was set at $p < 0.05$. All figures were performed using CorelDRAW X5 (Corel Corporation, Canada).

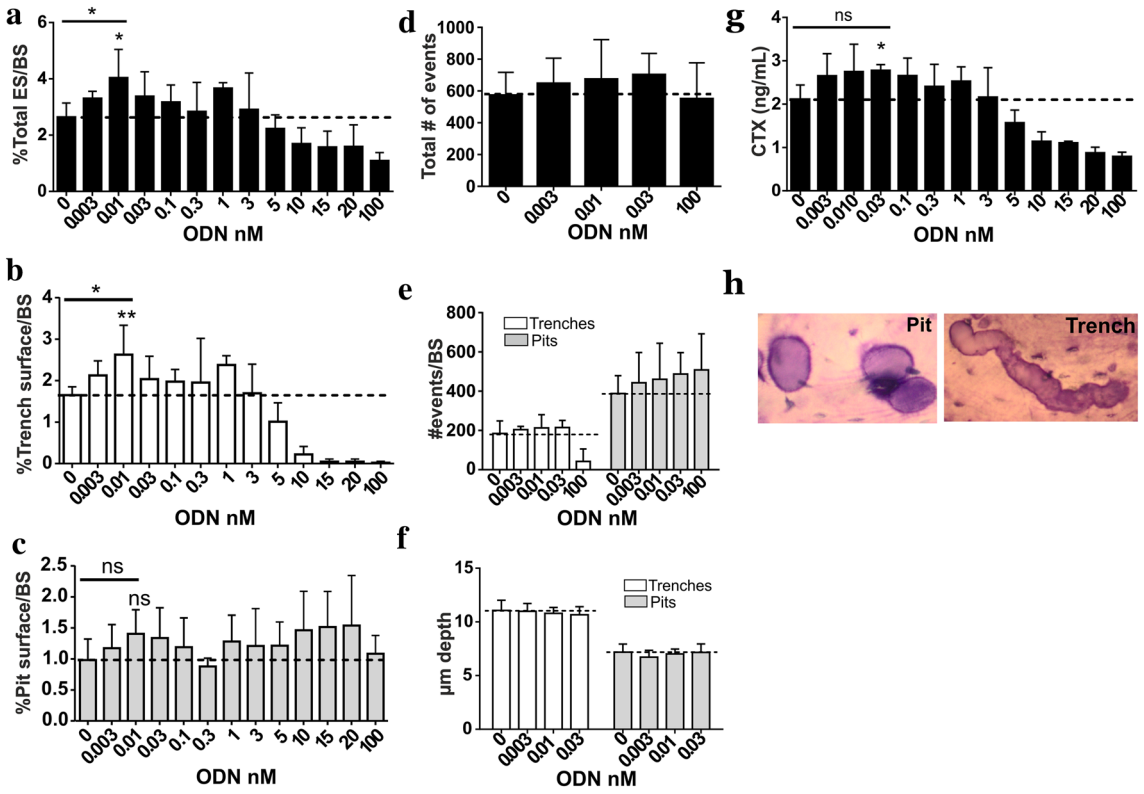

Fig. 1 Low doses of CatK inhibitor stimulate resorption by osteoclasts cultured on bone slices. Osteoclasts generated from a blood donor were cultured on bone slices in the presence of increasing doses of ODN ($n=4$ slices per condition). After 3 days, these slices were analyzed for total eroded surface per bone surface (**a**), trench surface per bone surface (**b**), pit surface per bone surface (**c**), total number of events per bone slice (**d**), number of events for trench and pit (**e**), maximum demineralization depth of trenches and pits (**f**), CTX levels in the conditioned media at the end of the culture (**g**). **h** Pictures illustrating the morphological appearance of a trench and a pit [30]. Dotted lines indicate the level of resorption in the absence of ODN. The statistical significance of the low-dose-induced stimulation of bone resorption was evaluated by the Kruskal–Wallis test, taking into account the doses ranging from 0 to the concentration of ODN where resorption reaches its nadir (**a** $*p=0.0410$, **b** $*p=0.0108$, and **g** $^{ns}p=0.1632$) and Dunn's multiple comparisons test (**a** $*p=0.0182$, **b** $**p=0.0092$, and **g** $*p=0.0376$)

Results

Low Doses of the Specific CatK Inhibitor ODN Stimulate Bone Resorption in Cultured Osteoclasts

Figure 1 shows the effect of increasing doses of ODN on osteoclast resorption, as assessed through a number of endpoints all obtained in the same experiment with osteoclasts from the same blood donor, and discriminating for different resorption patterns. At high doses (> 5 nM), these endpoints showed the typical responses reported earlier [8, 30–32]. The eroded surface was reduced (Fig. 1a), mainly as a result of complete suppression of trench formation (Fig. 1b), whereas the total number of events was not affected (Fig. 1d), because trench formation was replaced by pit formation (Fig. 1c, e). As expected, CTX, the collagen degradation product generated by CatK [33], was significantly decreased by high doses of ODN—just as trenches were. Importantly, in marked contrast with the effect of high doses of ODN, the low-dose 0.03 nM induced a significant increase in CTX, total eroded surface, and trench surface (Fig. 1a, b, g), but neither in trench number or in trench depth (Fig. 1e, f). There was also a general trend to an increase in pit surface and numbers, but not reaching statistical significance. Overall, this experiment indicates that ODN may affect osteoclast activity in two opposite ways—as particularly well shown by trench formation or CTX: at high dose, ODN acts as a strong antagonist as reported in the literature, but at low dose, it leads to a paradoxical stimulatory effect which has not received attention up to now.

The Low-Dose-Induced Stimulation is a General Characteristic of Human Osteoclasts

Next we asked how reproducible the "low-dose-induced" trench formation would be, by repeating the dose–response curve shown in Fig. 1b with osteoclasts obtained from 12 different blood donors (Fig. 2). We found that all 12 donors

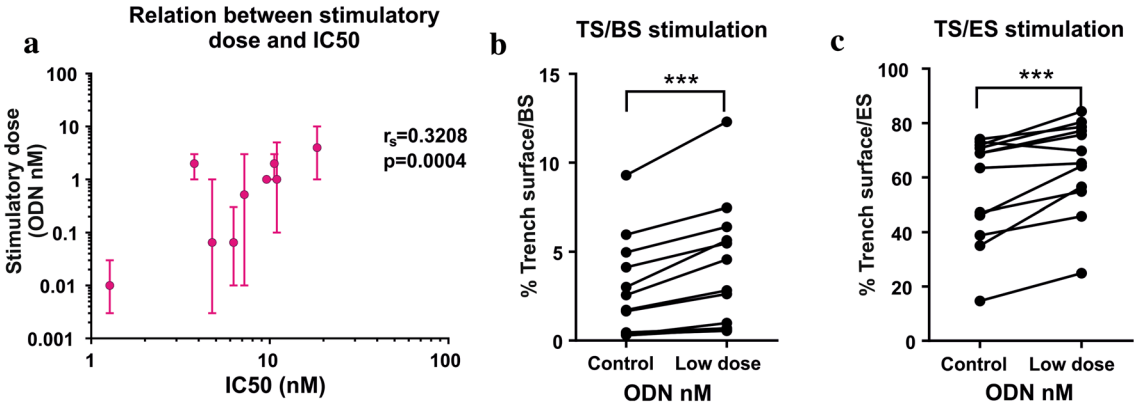

Fig. 2 Characteristics of the low-dose-induced stimulation assessed in osteoclast preparations of different donors. **a** The level of the low dose that stimulates resorption varies among osteoclasts generated from different donors, and this variation correlates with the corresponding IC50s for inhibition of trench formation. Dose–response curves as shown in Fig. 1a were repeated on preparations of nine different blood donors ($n = 3$–5 bone slices per dose for each experiment). For each dose–response curve, the range of the doses stimulating resorption, their medians, as well as the IC50 for inhibition of resorption were noted. The medians of stimulatory doses (medians shown as dots and full range shown as vertical lines) were plotted against the respective IC50s, and their relation was analyzed by using Spearman's test. **b**, **c** The degree of stimulation of trench surface/BS (**b**) and trench surface/ES (**c**) varies among osteoclasts generated from different blood donors ($n = 12$). For each of the dose–response curves such as used in (**a**), the control trench surface and the corresponding highest low-dose-induced trench surface were noted (shown as symbols connected by a line) and compared by using paired *t* test (***$p < 0.001$)

showed stimulated trench formation at low doses (Fig. 2b), and that trench formation increased more than pit formation (Fig. 2c). The degree of this stimulation varied from 1.25- to 3.2-fold, depending on the experiment. Furthermore, these different dose–response curves revealed that the actual doses inducing this stimulation varied among the blood donors, as did also the IC50s for inhibition of trench formation (Fig. 2a). Interestingly, when plotting the stimulatory doses of ODN measured for each blood donor against the respective IC50s, we found a correlation (Fig. 2a). This correlation indicates that the dose range inducing stimulation depends on the level of CatK activity in osteoclasts generated from a given osteoclast donor. Note in this respect that CatK expression has previously already been reported to vary substantially among donors [28] and to correlate with the proportion of eroded surface appearing as trenches [30]. In conclusion, the stimulation of osteoclast in response to a slight inhibition of CatK by ODN is a general osteoclast property, but the effective dose of ODN varies with the levels of CatK activity in osteoclast preparations.

The Low-Dose-Induced Stimulation Requires Interplay Between CatK and Other Cysteine Proteinases

A low dose of L873724, another specific CatK inhibitor [26, 34], proved to stimulate trench formation in the same way as ODN (not shown), thus confirming that this stimulation is induced through an effect on CatK. In contrast, general cysteine proteinase inhibitors such as E64 and K11017 [27] were not able to stimulate the osteoclasts (not shown), therefore leading to the hypothesis that the stimulatory effect requires the interplay between CatK and other cysteine proteinases. In order to test this hypothesis, we repeated dose–response curves with ODN as done for Figs. 1 and 2, but in the presence or absence of K11097, which is an inhibitor of all cysteine proteinases except CatK and does not inhibit bone resorption in control conditions [27] (Fig. 3a, b). These data showed that low doses of ODN were only able to stimulate osteoclasts in the absence of K11097, thus supporting that this stimulation requires interactions between CatK and other cysteine proteinases. In contrast, in the presence of GM6001, an inhibitor of MMPs, the low dose of ODN was still able to stimulate significantly the osteoclasts, thus suggesting that this stimulation does not require MMP activity of the osteoclasts (Fig. 3c, d). The data of Fig. 3 were reproduced with osteoclasts from another two blood donors.

Interplay Between CatK and Other Cysteine Proteinases for Degradation of Collagen in Enzymatic Assays

The absence of the low-dose-induced stimulation in the presence of non-CatK cysteine proteinase inhibitor in osteoclast culture prompted us to investigate whether other cysteine proteinases could contribute to CatK-induced collagen fiber degradation in test tube assays. Osteoclasts synthesize cysteine proteinases such as CatB and CatL, although at a lower level compared with CatK, as assessed in control

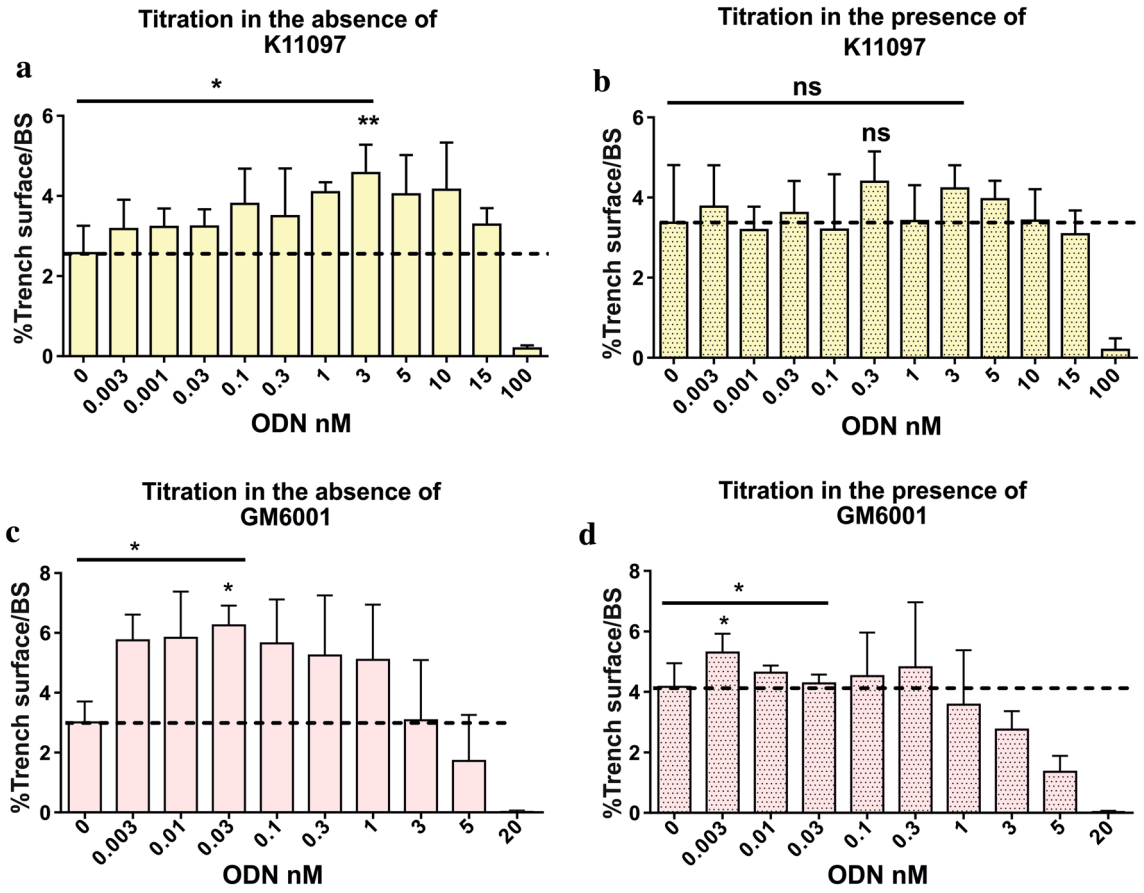

Fig. 3 The low-dose-induced stimulation of trench formation is prevented in the presence of non-CatK cysteine proteinase inhibitors, but not in the presence of MMP inhibitors. The response of trench formation to increasing doses of ODN was assessed in osteoclast preparations of two blood donors (**a, b** and **c, d**, respectively) either in the absence (**a, c**) or in the presence of 1 µM K11097 (inhibitor of Cat L, B, and S, but not of CatK) (**b**) or 6 µM GM6001 (inhibitor of MMPs) (**d**). The dotted line indicates the level of trench surface per bone surface in the absence of ODN. The statistical significance of the low-dose effect on trench formation was evaluated by using the Kruskal–Wallis test, taking into account the doses ranging from 0 to the dose where resorption reaches its nadir in (**a**) and (**c**) (**a** $*p=0.0276$, **b** $^{ns}p=0.7281$, **c** $*p=0.0197$, and **d** $*p=0.0441$) and Dunn's multiple comparisons test (**a** $**p=0.0011$, **b** $^{ns}p=0.3557$, **c** $*p=0.0136$, and **d** $*p=0.0213$)

conditions [35, 36]. Whereas CatK degrades collagen fibers through cleavage at multiple sites, including in the triple helix, CatB and CatL's cleavage sites are limited to the telopeptides, proteoglycan bridges, and unwound triple helices and have therefore a poor collagenolytic activity [19]. The data of Fig. 4 are in accordance with these properties. SEM examination of control collagen fibers showed compact structures (diameter, 48 ± 7.8 µm) with parallel arrangement of fibrils (~200 nm) with typical D-banding pattern (Fig. 4a). However, 6-h CatK-treated samples showed the splitting of collagen fibers (diameter, 48 ± 7.8 µm) into small fibril bundles (diameter, 4.0 ± 1.5 µm) (Fig. 4b). Upon incubation of the collagen fibers with CatB or CatL alone or in combination, these fibers remain packed although less tightly (increased diameter ~ 30%), and their surface becomes irregular (Fig. 4c–e). These changes presumably result from the cleavage of proteoglycan bridges [18]. However, if simultaneously incubated with CatK and CatB or CatL (each at a fivefold lower concentration than CatK), the collagen fibers become more dissociated into even smaller individual fibrils (50–200 nm) (Fig. 4 f–h). These different degrees of damage revealed by SEM are supported by the quantification of collagen fragments in the incubation medium (Fig. 4i). These data show that CatB and CatL can contribute to CatK-induced collagen fiber degradation, despite the strong collagenolytic power of CatK.

A Low Dose of ODN May Increase the Levels of CatB

Next, we wondered whether the involvement of non-CatK cysteine proteinases in the low-dose stimulation of resorption would go along with increased levels of these proteinases in osteoclast extracts. As shown in Fig. 2a, the low dose inducing a stimulation of resorption varies among donors,

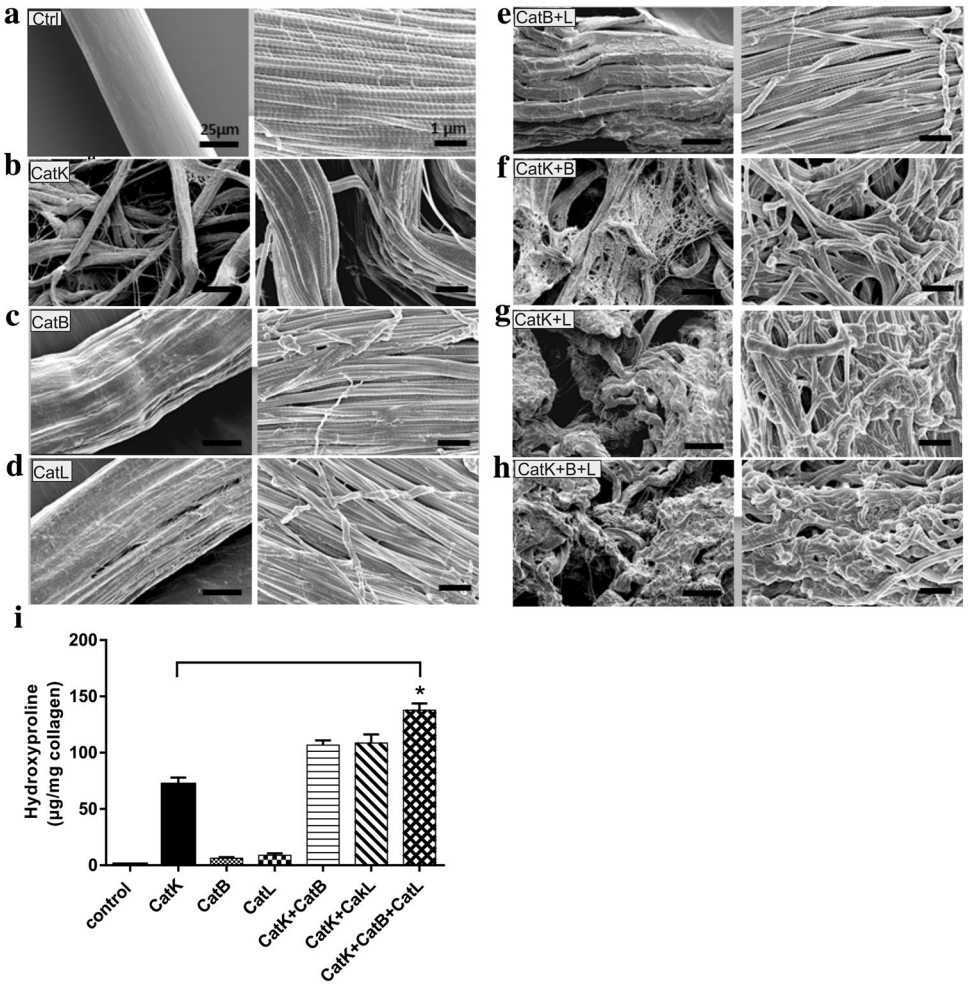

Fig. 4 Interplay between CatK and other cathepsins for degradation of collagen fibers. SEM images of collagen fibers untreated (**a**) and treated with CatK (**b**), CatL (**c**), CatB (**d**), CatL + B (**e**), CatK + L (**f**), CatK + B (**g**), CatK + L + B (**h**) in the conditions explained in the methods. Left panel demonstrates the morphology of collagen fibers (bars represent 25 µm) and right panel shows the magnified surface view of fibers (bars represent 1 µm). **i** Evaluation of collagen degradation by measuring hydroxyproline in supernatants harvested after the incubations described in Fig. 5a–h and then hydrolyzed. The addition of low concentrations of CatB and L to CatK shows a significant increase in collagen degradation, as evaluated with a *t* test (*$p < 0.028$)

and cannot be known in advance. These experiments thus demand both a dose–response curve monitoring resorption as in Fig. 1 and the level of active cathepsins in the osteoclast extract by using Western blots for example. Together, this thus demands a substantial amount of osteoclasts from each donor. Nevertheless, three such experiments were performed by using osteoclasts each from a different donor (Fig. 5). The Western blots showed that CatB and CatL antibodies detected bands at the expected molecular mass of the active and the precursor forms of human CatB and CatL [37, 38] (Fig. 5a, c). Of note, the intensity of the bands corresponding to active CatB were increased about 1.5 times at the low dose of ODN in all three experiments, but CatL was increased in only one of them.

Discussion

The present study provides a possible explanation for why a slight inhibition of CatK stimulates bone loss as observed in vivo [10]. A major result is that the osteoclast alone is enough to show stimulation without requiring any other cell type. Incidental data of Leung et al. already suggested this possibility, as ODN at a dose of 1 nM induced a mild increase of the bone resorption marker CTX in the conditioned media of human osteoclasts cultured on bone slices [31]. The present study represents a definitive demonstration, based on repeated titrations curves each performed with osteoclasts obtained from different donors. These titrations were indispensable for our demonstration because of the great inter-individual variation in the dose stimulating resorption (Fig. 2a). At first sight, this variation appears surprising, but we found that it coincides with the variation in the levels of osteoclastic CatK activity among different individuals assessed here through IC50 (Fig. 2a). The reason for this coincidence remains to be investigated. Note that a recent study had already called the attention on the greater variation in CatK mRNA expression in osteoclasts prepared from different individuals, compared with other osteoclast proteinases [28].

Our demonstration of the low-dose-induced stimulation is further strengthened by the fact that it is supported by independent endpoints: erosion and CTX. The actual number of resorption events is not affected by the low doses

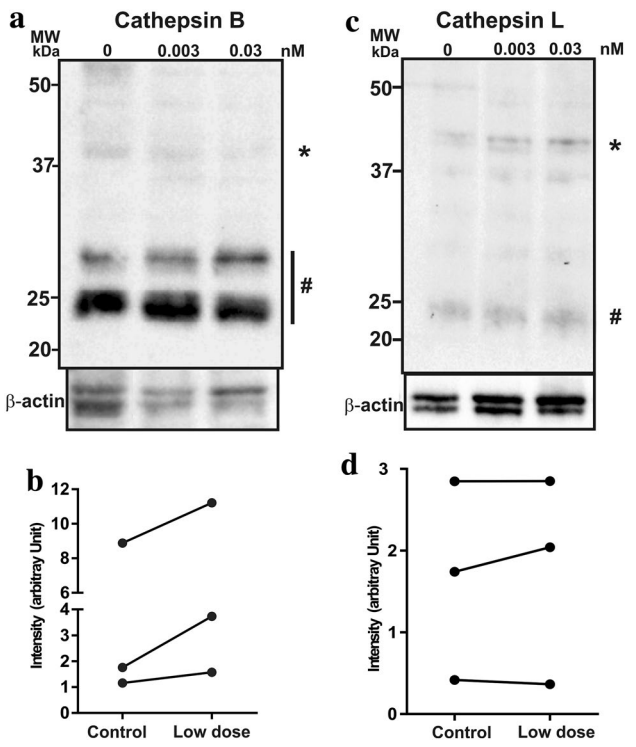

Fig. 5 Effect of a mild inhibition of CatK on the levels of CatB and L. Osteoclast preparations from three different donors were cultured in the presence of increasing doses of ODN. For each preparation, the specific dose inducing the highest TS/BS level was determined. The osteoclasts subjected to this specific dose and the corresponding control osteoclasts were lysed. These lysates were then analyzed for the levels of CatB and CatL through Western blot. Representative Western blots of CatB (**a**) and CatL (**c**) in extracts of osteoclasts of one of the donors, cultured in the presence of the indicated doses of ODN. The bands corresponding to pro-cathepsin (*) and active cathepsin (#) were identified. **b, d** Intensity levels of the bands corresponding to the active CatB and CatL in all three pairs of control osteoclasts and osteoclasts subjected to low-dose-stimulated resorption (normalized to beta-actin)

inducing elevated erosion and CTX, thereby showing that the stimulatory mechanism is not caused by increased activation frequency of resorption, but rather by extension of resorption of each event. Here this extension is not brought about by increasing depths, but mostly by enlargement of the surface of the excavations. This enlargement rather results from formation of long trenches than of round pits, as shown by the low-dose-induced increased proportion of trenches in 12 different experiments. These morphological details of excavations have some importance, because high collagen degradation is an absolute prerequisite for trench formation—but not for pit formation [30]. Furthermore, the absence of collagen leftover is a hallmark of trenches, in contrast with abundant collagen leftover in pits [8]. Thus, these excavation characteristics point to enhanced collagenolysis as responsible of stimulation of resorption by a mild inhibition of CatK. However, how can a mild "inhibition" of the main collagenolytic osteoclast proteinase lead to overall "enhanced" collagenolysis? What is the mechanism?

A hint to this mechanism is that K11097, an inhibitor of non-CatK cysteine proteinases, prevents the low-dose stimulation induced by ODN, thus indicating that the low-dose-induced collagenolytic power results from the interplay between CatK and other cysteine proteinases. The participation of other cysteine proteinases in collagen degradation may appear astonishing at first sight, since CatK appears the only essential collagenase of osteoclasts under physiological conditions [1, 4, 6]. However, pycnodysostosis or CatK-knockout experiments prove that skeletal development is still possible in the absence of CatK, and prove therefore that other collagenolytic proteinases can to some extent rescue the tasks of CatK [6, 12–14]. Collagenolytic MMPs and non-CatK cysteine proteinases were proposed [1]. Regarding MMPs, osteoclasts produce high levels of MMP9 and MT1-MMP [20], and their involvement in given situation of resorption has been considered [1, 39, 40]. However, herein the MMP inhibitor GM6001 does not prevent the low-dose stimulation, suggesting that MMP activity is not involved in this stimulation. Furthermore, the levels of these MMPs in extracts of osteoclasts subjected to a low dose were not significantly increased (not shown) as they are in osteoclasts where CatK activity is completely prevented [13]. Still, one may wonder whether at the bone tissue level, osteoblast MMPs might also contribute to the low-dose stimulation, as several and diverse observations point to their role in osteoclast resorption [12, 20, 40]. The answer to this question is awaiting low-dose tests in cultures of bone explants or osteoclast–osteoblast co-cultures. Regarding non-CatK cysteine proteinases, silencing osteoclastic cysteine proteinases such as CatB or CatL does not affect bone resorption [14, 27, 41, 42]. However, there are cell types where CatB appears responsible for strong collagenolysis [43, 44], and there are situations where CatB and L to some extent can contribute to osteoclast resorptive activity [14, 45]. The present study indicates that the latter may also apply to the low-dose stimulation, not only because it is abolished by an inhibitor of non-CatK cysteine proteinases, but also (i) because our enzymatic assays of collagen fiber degradation show that CatB and L may add to the degradation exerted by the very potent CatK, and (ii) because our Western blots of osteoclast extracts indicate a low-dose-induced increase in CatB levels and sometimes in CatL levels. The latter observation means that the balance between the production and degradation of CatB is shifted to a higher steady-state level. Increased production [13] and half-life [11, 16] of lysosomal proteinases have both been reported in situations of cysteine proteinase inhibition, and it remains to be investigated which of these alternatives is involved in the present low-dose effect.

An important question is how, at the molecular level, common proteinases like CatB and L can supply extra

collagenolytic power to CatK when the collagenolytic efficiency of CatK is far beyond that of all other known proteinases [18, 19]. Degradation of collagen fibers demands successive cleavages of different types [18, 46]: (i) cleavages of proteoglycan bridges for disassembling the collagen fibrils; (ii) cleavages of telopeptides to start the loosening process of collagen triple helix units; (iii) cleavages of the triple helix at multiple sites for full release and unwinding of the triple helix—which requires very specialized enzymatic characteristics; and (iv) final cleavages of the collagen chain fragments into small peptides. Of note, each of these steps differs in terms of peptide bonds to be cleaved and accessibility, and has therefore distinct demands. It is remarkable that CatK acts like a multiple purpose tool that can alone exert all these activities. This versatility results from its ability to form diverse supramolecular complexes specifically adapted to the peculiarities of the respective cleavage steps [46]. On the other hand, common proteinases like CatB and L are able to contribute to all cleavage steps except the cleavages of the triple helix [18, 19, 47], and it is thus by doing so that these proteinases provide extra overall collagenolytic power. This view is supported by our observations that simultaneous activity of CatK and B or L results in increased collagenolytic power compared with that of CatK alone, and that the low-dose-induced collagenolysis requires the simultaneous activity of CatK and other cysteine proteinases.

In conclusion, we show herein that the enzymatic machinery of the osteoclast alone is enough to respond to a mild inhibition of CatK by increased resorptive activity. This mimics the ODN-induced increase of CTX in the phase 2 clinical trial [10]. In addition, it shows that the mechanism of this stimulation is based on an interaction between CatK and other cysteine proteinases. Our observations highlight once more that collagenolysis is very critical for the regulation of bone resorption levels [9, 25, 30], and therefore a critical target for anti-resorptive drugs.

Acknowledgements We thank Jacob Bastholm Olesen for excellent technical assistance and Anne V Schmedes and Merete Villiumsen for her kind assistance on biochemical procedures. This study has received financial support from The Region of Southern Denmark (Grant No. 13/27663), Vejle Hospital/Lillebaelt Hospital and the University of Southern Denmark.

Author Contributions Designing the study: DCP, KS, and J-MD; conducting experiments: DCP, PP, and KS; acquiring data: DCP, PP, MO, MLB, JSM, and KS; analyzing data: DCP, PP, DB, MO, KS, and J-MD; writing the manuscript: DCP, KS, and J-MD; editing and correcting the manuscript: all authors; final approval of manuscript: all authors.

Compliance with Ethical Standards

Conflict of interest Dinisha Cyril Pirapaharan, Kent Søe, Preety Panwar, Jonna Skov Madsen, Marianne Lerbæk Bergmann, Martin Overgaard, Dieter Brömme, and Jean-Marie Delaisse declare that they have no conflict of interest.

Ethical Approval The study has been performed in accordance with the ethical standards and was approved by The Regional Committees on Health Research Ethics for Southern Denmark (approval number. 2007–0019).

Informed Consent All participants in this study provided written informed consent.

References

1. Delaisse JM, Andersen TL, Engsig MT, Henriksen K, Troen T, Blavier L (2003) Matrix metalloproteinases (MMP) and cathepsin K contribute differently to osteoclastic activities. Microsc Res Tech 61:504–513
2. Saftig P, Hunziker E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W et al (1998) Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. Proc Natl Acad Sci USA 95:13453–13458
3. Gelb BD, Moissoglu K, Zhang J, Martignetti JA, Bromme D, Desnick RJ (1996) Cathepsin K: isolation and characterization of the murine cDNA and genomic sequence, the homologue of the human pycnodysostosis gene. Biochem Mol Med 59:200–206
4. Drake MT, Clarke BL, Oursler MJ, Khosla S (2017) Cathepsin K inhibitors for osteoporosis: biology, potential clinical utility, and lessons learned. Endocr Rev 38:325–350
5. Duong LT (2012) Therapeutic inhibition of cathepsin K-reducing bone resorption while maintaining bone formation. Bonekey Rep 1:67
6. Bromme D, Panwar P, Turan S (2016) Cathepsin K osteoporosis trials, pycnodysostosis and mouse deficiency models: commonalities and differences. Expert Opin Drug Discov 11:457–472
7. Mukherjee K, Chattopadhyay N (2016) Pharmacological inhibition of cathepsin K: a promising novel approach for postmenopausal osteoporosis therapy. Biochem Pharmacol 117:10–19
8. Panwar P, Soe K, Guido RV, Bueno RV, Delaisse JM, Bromme D (2016) A novel approach to inhibit bone resorption: exosite inhibitors against cathepsin K. Br J Pharmacol 173:396–410
9. Panwar P, Xue L, Soe K, Srivastava K, Law S, Delaisse JM et al (2017) An ectosteric inhibitor of cathepsin K inhibits bone resorption in ovariectomized mice. J Bone Miner Res 32:2415–2430
10. Bone HG, McClung MR, Roux C, Recker RR, Eisman JA, Verbruggen N et al (2010) Odanacatib, a cathepsin-K inhibitor for osteoporosis: a two-year study in postmenopausal women with low bone density. J Bone Miner Res 25:937–947
11. Montenez JP, Delaisse JM, Tulkens PM, Kishore BK (1994) Increased activities of cathepsin B and other lysosomal hydrolases in fibroblasts and bone tissue cultured in the presence of cysteine proteinases inhibitors. Life Sci 55:1199–1208
12. Everts V, Delaisse JM, Korper W, Jansen DC, Tigchelaar-Gutter W, Saftig P et al (2002) The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. J Bone Miner Res 17:77–90
13. Kiviranta R, Morko J, Alatalo SL, NicAmhlaoibh R, Risteli J, Laitala-Leinonen T et al (2005) Impaired bone resorption in cathepsin K-deficient mice is partially compensated for by enhanced osteoclastogenesis and increased expression of other proteases via an increased RANKL/OPG ratio. Bone 36:159–172
14. Everts V, Korper W, Hoeben KA, Jansen ID, Bromme D, Cleutjens KB et al (2006) Osteoclastic bone degradation and the role of different cysteine proteinases and matrix metalloproteinases: differences between calvaria and long bone. J Bone Miner Res 21:1399–1408

15. Sutherland JH, Greenbaum LM (1983) Paradoxical effect of leupeptin in vivo on cathepsin B activity. Biochem Biophys Res Commun 110:332–338
16. Kominami E, Tsukahara T, Bando Y, Katunuma N (1987) Autodegradation of lysosomal cysteine proteinases. Biochem Biophys Res Commun 144:749–756
17. Tanaka K, Ikegaki N, Ichihara A (1981) Effects of leupeptin and pepstatin on protein turnover in adult rat hepatocytes in primary culture. Arch Biochem Biophys 208:296–304
18. Panwar P, Du X, Sharma V, Lamour G, Castro M, Li H et al (2013) Effects of cysteine proteases on the structural and mechanical properties of collagen fibers. J Biol Chem 288:5940–5950
19. Garnero P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS et al (1998) The collagenolytic activity of cathepsin K is unique among mammalian proteinases. J Biol Chem 273:32347–32352
20. Andersen TL, del Carmen OM, Kirkegaard T, Lenhard T, Foged NT, Delaisse JM (2004) A scrutiny of matrix metalloproteinases in osteoclasts: evidence for heterogeneity and for the presence of MMPs synthesized by other cells. Bone 35:1107–1119
21. Engsig MT, Chen QJ, Vu TH, Pedersen AC, Therkidsen B, Lund LR et al (2000) Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. J Cell Biol 151:879–889
22. Ohuchi E, Imai K, Fujii Y, Sato H, Seiki M, Okada Y (1997) Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. J Biol Chem 272:2446–2451
23. Panwar P, Butler GS, Jamroz A, Azizi P, Overall CM, Bromme D (2018) Aging-associated modifications of collagen affect its degradation by matrix metalloproteinases. Matrix Biol 65:30–44
24. Moller AM, Delaisse JM, Soe K (2017) Osteoclast fusion: time-lapse reveals involvement of CD47 and syncytin-1 at different stages of nuclearity. J Cell Physiol 232:1396–1403
25. Soe K, Delaisse JM (2010) Glucocorticoids maintain human osteoclasts in the active mode of their resorption cycle. J Bone Miner Res 25:2184–2192
26. Desmarais S, Masse F, Percival MD (2009) Pharmacological inhibitors to identify roles of cathepsin K in cell-based studies: a comparison of available tools. Biol Chem 390:941–948
27. Xia L, Kilb J, Wex H, Li Z, Lipyansky A, Breuil V et al (1999) Localization of rat cathepsin K in osteoclasts and resorption pits: inhibition of bone resorption and cathepsin K-activity by peptidyl vinyl sulfones. Biol Chem 380:679–687
28. Merrild DM, Pirapaharan DC, Andreasen CM, Kjaersgaard-Andersen P, Moller AM, Ding M et al (2015) Pit- and trench-forming osteoclasts: a distinction that matters. Bone Res 3:15032
29. Bromme D, Nallaseth FS, Turk B (2004) Production and activation of recombinant papain-like cysteine proteases. Methods 32:199–206
30. Soe K, Merrild DM, Delaisse JM (2013) Steering the osteoclast through the demineralization-collagenolysis balance. Bone 56:191–198
31. Leung P, Pickarski M, Zhuo Y, Masarachia PJ, Duong LT (2011) The effects of the cathepsin K inhibitor odanacatib on osteoclastic bone resorption and vesicular trafficking. Bone 49:623–635
32. Zhuo Y, Gauthier JY, Black WC, Percival MD, Duong LT (2014) Inhibition of bone resorption by the cathepsin K inhibitor odanacatib is fully reversible. Bone 67:269–280
33. Garnero P, Ferreras M, Karsdal MA, NicAmhlaoibh R, Risteli J, Borel O et al (2003) The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation. J Bone Miner Res 18:859–867
34. Li CS, Deschenes D, Desmarais S, Falgueyret JP, Gauthier JY, Kimmel DB et al (2006) Identification of a potent and selective non-basic cathepsin K inhibitor. Bioorg Med Chem Lett 16:1985–1989
35. Drake FH, Dodds RA, James IE, Connor JR, Debouck C, Richardson S et al (1996) Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. J Biol Chem 271:12511–12516
36. Ishibashi O, Inui T, Mori Y, Kurokawa T, Kokubo T, Kumegawa M (2001) Quantification of the expression levels of lysosomal cysteine proteinases in purified human osteoclastic cells by competitive RT-PCR. Calcif Tissue Int 68:109–116
37. Moin K, Day NA, Sameni M, Hasnain S, Hirama T, Sloane BF (1992) Human tumour cathepsin B. Comparison with normal liver cathepsin B. Biochem J 285(Pt 2):427–434
38. Reilly JJ Jr, Mason RW, Chen P, Joseph LJ, Sukhatme VP, Yee R et al (1989) Synthesis and processing of cathepsin L, an elastase, by human alveolar macrophages. Biochem J 257:493–498
39. Delaisse JM, Boyde A, Maconnachie E, Ali NN, Sear CH, Eeckhout Y et al (1987) The effects of inhibitors of cysteine-proteinases and collagenase on the resorptive activity of isolated osteoclasts. Bone 8:305–313
40. Everts V, Delaisse JM, Korper W, Niehof A, Vaes G, Beertsen W (1992) Degradation of collagen in the bone-resorbing compartment underlying the osteoclast involves both cysteine-proteinases and matrix metalloproteinases. J Cell Physiol 150:221–231
41. Potts W, Bowyer J, Jones H, Tucker D, Freemont AJ, Millest A et al (2004) Cathepsin L-deficient mice exhibit abnormal skin and bone development and show increased resistance to osteoporosis following ovariectomy. Int J Exp Pathol 85:85–96
42. James IE, Marquis RW, Blake SM, Hwang SM, Gress CJ, Ru Y et al (2001) Potent and selective cathepsin L inhibitors do not inhibit human osteoclast resorption in vitro. J Biol Chem 276:11507–11511
43. Lee ER, Lamplugh L, Shepard NL, Mort JS (1995) The septoclast, a cathepsin B-rich cell involved in the resorption of growth plate cartilage. J Histochem Cytochem 43:525–536
44. Creemers LB, Hoeben KA, Jansen DC, Buttle DJ, Beertsen W, Everts V (1998) Participation of intracellular cysteine proteinases, in particular cathepsin B, in degradation of collagen in periosteal tissue explants. Matrix Biol 16:575–584
45. Hill PA, Buttle DJ, Jones SJ, Boyde A, Murata M, Reynolds JJ et al (1994) Inhibition of bone resorption by selective inactivators of cysteine proteinases. J Cell Biochem 56:118–130
46. Aguda AH, Panwar P, Du X, Nguyen NT, Brayer GD, Bromme D (2014) Structural basis of collagen fiber degradation by cathepsin K. Proc Natl Acad Sci USA 111:17474–17479
47. Maciewicz RA, Wotton SF, Etherington DJ, Duance VC (1990) Susceptibility of the cartilage collagens types II, IX and XI to degradation by the cysteine proteinases, cathepsins B and L. FEBS Lett 269:189–193