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Non-Invasive Optical Detection of Cathepsin K-Mediated Fluorescence Reveals Osteoclast Activity In Vitro and In Vivo

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Abstract

Osteoclasts degrade bone matrix by demineralization followed by degradation of type I collagen through secretion of the cysteine protease, cathepsin K. Current imaging modalities are insufficient for sensitive observation of osteoclast activity, and in vivo live imaging of osteoclast resorption of bone has yet to be demonstrated. Here, we describe a near-infrared fluorescence reporter probe whose activation by cathepsin K is shown in live osteoclast cells and in mouse models of development and osteoclast upregulation. Cathepsin K probe activity was monitored in live osteoclast cultures and correlates with cathepsin K gene expression. In ovariectomized mice, cathepsin K probe upregulation precedes detection of bone loss by micro-computed tomography. These results are the first to demonstrate non-invasive visualization of bone degrading enzymes in models of accelerated bone loss, and may provide a means for early diagnosis of upregulated resorption and rapid feedback on efficacy of treatment protocols prior to significant loss of bone in the patient.

Keywords

osteoclast; cathepsin K; non-invasive imaging; molecular imaging; fluorescence

INTRODUCTION

Osteoclasts are responsible for the destruction and removal of bone in the skeleton, and a proper balance between osteoblastic bone formation and osteoclastic resorption is critical for maintenance of bone structure and function. The primary components of bone matrix are removed by osteoclasts first by demineralization of the inorganic mineral through acidification of the local bone microenvironment followed by degradation of collagen by the cysteine protease, cathepsin K [1–3]. Upregulation of osteoclast activity is associated with osteoporosis,

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metastasis-induced osteolysis, Paget's disease, rheumatoid arthritis, and periodontal disease, while mutations in the genes encoding for cathepsin K result in inability of the osteoclast to degrade type I collagen, as seen in knockout mice and the human condition pycnodysostosis [4–6].

Current skeletal imaging techniques and biochemical markers of bone metabolism fail to combine spatial information with indicators of cellular activity. Urine and serum biochemical markers for pyridinolines, collagen telopeptides, and TRACP5b provide sensitive measures of bone destruction,[7] but cannot localize osteoclast upregulation to distinct anatomic location. Imaging modalities such as X-ray, DXA, and pQCT report bone mass and tissue organization, representing the integrated effects of osteoclast and osteoblast activity, whereas therapeutic interventions primarily alter one or the other. Moreover, the appearance of altered skeletal structure lags behind actual cellular activity, representing a delay of information for the clinician [8]. The development of image-based indicators of osteoclast activity will allow for early identification of changes in bone resorption. Treatment options based on early detection of upregulated osteoclast activity rather than downstream global changes in bone mass will help clinicians better identify proper interventions as well as allow for real-time feedback on drug efficacy to better monitor drug dose and interval.

To date, live in vivo imaging of osteoclasts has been limited to radiolabeled ligands specific for the vitronectin receptor present in osteoclasts [9]. In vitro, osteoclasts have been modified to induce promoter-driven fluorescence, and the interactions of cells with various lipophilic-enhanced fluorescent markers has been studied [10–12]. However, none of these techniques directly report osteoclast catabolic activity. Fluorescence-based imaging relying on enzyme-specific protease activation of fluorescence has been shown to denote cellular activity in a spatially sensitive context in models of cancer, atherosclerosis, and arthritis, among others [13–15]. Here, we demonstrate live cell imaging of cathepsin K-activated fluorescence in osteoclast cultures and in vivo models of bone loss. Cathepsin K-induced fluorescence precedes detection of bone loss by standard radiographic techniques, and may represent a new screening tool for monitoring early changes in osteoclast upregulation.

MATERIALS AND METHODS

NIRF probes

The near-infrared cathepsin K probe (CatK) consists of an MPEG d-poly-lysine amino acid backbone chain functionalized with Cy5.5 fluorophores through the cathepsin K-sensitive link sequence GHPG-GPQGKC [16]. In its native state, fluorophores are optically quenched due to proximity to one another, however upon cleavage, fluorophores are released and fluorescence increases. Previous enzymatic studies have shown that cathepsin K enzyme preferentially activates the CatK probe compared to cathepsins B and L, and MMP's -2 and -9 [17]. CatK and d-control near-infrared fluorescent probes were synthesized as previously described [17]. The far-red fluorescent pamidronate, Osteosense750 (FRFP750; VisEn Medical Inc., Woburn MA) consists of a bisphosphonate linked to a fluorophore and allows for non-invasive monitoring of new bone formation in an optical channel separate from the CatK probe [18]. Prosense680 (PS680; VisEn Medical Inc., Woburn MA) is an activatable fluorescent probe cleaved by cathepsins B, L, S and plasmin [14].

Imaging Modalities

Plate reader assays were performed on a Tecan Safire² microplate system. In vitro imaging was performed on a near-infrared intravital laser scanning microscope (Olympus IV-100) capable of simultaneous multichannel imaging [19] (excitation/emission filter combinations GFP: 488 nm/515±10nm, CY5.5: 633 nm/695±35nm, AF750 748 nm/770 nm LP). In vivo and

ex vivo fluorescence reflectance imaging (FRI) was performed using a modified bonSAI system (Siemens Medical Solutions; excitation/emission filter set: CY5.5: 635±15nm/695±15nm, AF750: 740±20nm/800±20nm). In vivo fluorescence molecular tomography (FMT) was performed using a VisEn FMT system (VisEn Medical Inc., Woburn MA; excitation laser/emission filter 670/700 nm, 745/775 nm). Fluorescence microscopy was performed with a Nikon 80i Eclipse microscope with appropriate filter sets (excitation/emission UV:365nm/400nm LP, CY5.5: 650±45nm/710±50nm, AF750: 775±50nm/845±55nm). Micro-computed tomography was carried out with a GE/EVS MS-8 cone beam small specimen μ QCT scanner (GE Healthcare Biosciences, London Ontario, Canada).

Enzyme Assays

Cleavage of fluorescent probes was assessed in triplicate by plate reader assay in total volumes of 100 μ L. Recombinant human cathepsin K (370 nM, Calbiochem) or mouse cathepsin K (424 nM, kind gift of Medivir UK Ltd.) was added to CatK, d-control, or PS680 probe (1 μ M). Cathepsin K activity was inhibited by the cathepsin K inhibitor 1,3-Bis(CBZ-Leu-NH)-2-propanone (2.1 mM, Calbiochem). Trypsin (1 mU, Sigma) specifically cleaves Lys-Lys bonds, and was used to test specificity of cathepsin K cleavage site and stability of CatK and d-control amino acid backbone sequences. Cathepsin K enzyme and inhibitor were prepared in a buffer of 0.1M phosphate, 1mM EDTA, 2mM DTT, at pH 6.0 as described elsewhere [16,17]. Trypsin was prepared in a buffer of 20 mM NaOAc, 1mM EDTA, 1 mM DTT, at pH 5.0. All experiments were performed in volumes of 100 μ L in 96 well plates.

In Vitro Culture

Raw 264.7 cells were grown in α MEM, supplemented with 10% FBS, 1% Pen-Strep, and 1% L-glutamine. Cells were induced to form osteoclasts by plating between 5000 and 10,000 cells/mL in 96 well plates on either plastic or sperm whale dentin surfaces and treating with 50 ng/mL hRANKL every 2–3 days. After 6–7 days of culture, cells were imaged. CatK probe or d-control probe were added at a concentration of 0.2 μ M between 4 and 20 hours prior to imaging. The nuclear label Syto24 (Invitrogen) was added approximately 1 hour prior to imaging (10 μ M). Cells were imaged live at 37°C at noted magnification.

In Vitro Cathepsin K Inhibition

Following 6 days of RANKL treatment, cells were pre-incubated with 10 μ M cathepsin K inhibitor for 15 minutes. After pre-incubation, CatK probe (0.2 μ M) and RANKL (50 ng/mL) were added to cathepsin K inhibitor, and cells were incubated with the probe-inhibitor mix for 2 hours. Control cells received CatK probe and RANKL but no cathepsin K inhibitor. Following 2 hours of culture, media was assayed for fluorescence activity via plate reader assay. In duplicate wells, RNA from cells on dentin and plastic, with and without cathepsin K inhibitor, was isolated and reverse transcribed to form cDNA. Amplification took place using cathepsin K primer sequences (Forward 5'-CTCACCAGAAGCAGTATAAC; Reverse 5'-TGG TTCATGGCCAGTTCATA). HPRT was used as a housekeeping control (Forward 5'-TCAAGGGCATATCGAACAACAAAC; Reverse 5'-AGCAGTACAGCCCCAAAATGGTTA).

In Vivo Imaging

Neonatal Mouse—To screen for areas of high probe activity, a developmentally immature 7 day old neonatal Tie2 mouse was administered 0.2 nmoles of CatK probe and FRFP750 by i.p. injection. 24 hours post-probe, the mouse was imaged via FRI. Paws, legs, feet and tail demonstrated high probe labeling and were subsequently dissected and re-imaged ex vivo for spatial co-localization of both probes.

Ovariectomized Mice—10 week female Balb/c mice (Charles River Laboratories) were subjected to either ovariectomy (OVX), OVX with daily pamidronate (0.5 mg/kg/day i.p.; OVX-Pam), or sham surgery. Mice were divided into two cohorts- short-term bone loss (STBL) and long-term bone loss (LTBL). On day 7 (STBL) and day 13 (LTBL) following surgery, mice were administered CatK probe (n=5/group). Two mice per group were given an additional injection of FRFP750 to monitor new bone formation simultaneously. 3 additional OVX mice in both STBL and LTBL groups were given d-control probe to demonstrate specificity of the cathepsin K cleavage sequence. All imaging doses consisted of 2 nmoles in 150 μ L sterile saline administered via tail vein injection.

Mice were imaged by FMT 20–22 hours post-probe injection. To minimize non-specific scattering of fluorophore, mouse hair was removed at the time of probe administration. During FMT scanning, mice were anesthetized via 2% isoflurane inhalation and positioned with tibiae centrally located in the scan field of view. 3D reconstruction was performed using vendor software with reconstruction size of approximately $1.0 \times 1.0 \times 0.5$ mm voxel size. Reconstructions were exported as DICOM image and analyzed using ImageJ software (National Institutes of Health). 10 mm regions of interest were isolated from each tibia extending distally from peak fluorescence at the proximal tibial growth plate. Total tibial fluorescence for each mouse was measured for each probe and compared. Following FMT imaging, mice were euthanized by CO₂ inhalation. Femora and tibiae were dissected into ice cold Hank's buffered salt solution (Mediatech Inc, Herndon VA) with 2% fetal calf serum to prepare for either ex vivo fluorescent imaging or μ QCT followed by histological examination.

μ QCT Bone Volume Analysis—Right tibiae were fixed in 10% NBF for 48 hours at 4°C. After fixation, tibiae were stored in 70% ETOH. Specimens were scanned by μ QCT in 70% ETOH and reconstructed at 18 μ m voxel size. Bone volume fraction of the proximal tibia distal to the growth plate was analyzed by manufacturer-provided software (MicroView, GE Healthcare Biosciences).

Fluorescence Microscopy—Left tibiae were embedded in 7% gelatin (Sigma) or OCT compound (Tissue-Tek) and snap frozen in a dry ice-isopentane slurry. 7 μ m non-decalcified sections were obtained using the CryoJane tape transfer cutting system (Instrumedics Inc., St. Louis MO). Fresh sections were imaged by fluorescence microscopy to visualize probe distribution within the tissue.

Cathepsin K Immunohistochemistry—Adjacent sections to those imaged by fluorescence microscopy were fixed in 4% paraformaldehyde and immunohistochemistry was performed using the avidin-biotin-peroxidase technique with rabbit polyclonal antibody for cathepsin K (Santa Cruz Biotech, Santa Cruz CA).

Ex Vivo Specimen Imaging—Following dissection, neonatal tail, arm and paw, and OVX femora were imaged by FRI. Neonatal tail was analyzed by plotting the fluorescence profile for FRFP750 and CatK probe along the tail length. OVX femora were analyzed by defining a region of interest extending from the lateral third trochanter to the distal femoral condyles. Transverse slices were assessed for total CatK and FRFP750 fluorescence, and location of peak fluorescence was noted for each probe. Fluorescence separation (FlSp) was determined for each sample by noting the absolute difference in spatial position between peak fluorescence of FRFP750 probe minus CatK probe and compared.

Statistics—Enzyme assays, ovariectomy fluorescence, and microCT were assessed by one-way ANOVA followed by Tukey post-hoc tests for significance between groups. Conditioned media fluorescence was assessed by two-way ANOVA with surface and inhibitor as factors. Data is presented as mean $\pm$ SD with significance attributed to $p < 0.05$.

RESULTS

Cathepsin K probe is cleaved at cathepsin K recognition site

Incubation of CatK probe with mouse or human cathepsin K enzyme results in a 5–6 fold increase in fluorescence over baseline and is inhibited by addition of the cathepsin K inhibitor 1,3-Bis(CBZ-Leu-NH)-2-propanone (Figure 1). In contrast, a control probe (d-control) differing from CatK probe only by inclusion of a non-cleavable d-enantiomer form of the cathepsin K-sensitive linker shows no activation, verifying reporter probe cleavage by cathepsin K is specific to the linker region only. Both CatK and d-control probes contain d-enantiomer poly-lysine backbones. To assess the resistance of d-poly-lysine to cleavage, probes were incubated with trypsin- a potent cleaver of L-poly-lysine bonds. Neither probe demonstrate fluorescence activation after 22 hours exposure to trypsin, compared to PS680, a conceptually similar probe that contains L-poly-lysine bonds as the activation site between fluorophores (Figure 1).

Osteoclasts reveal upregulated cathepsin K fluorescence in live cell imaging

RAW 264.7 cells cultured for 6–7 days in the presence of RANKL fuse to become osteoclasts, demonstrating a typical multi-nuclear appearance, TRAP-positive staining, and actin ring formation (Figure 2C, F). Osteoclasts demonstrate intracellular cathepsin K probe activation after incubation with probe (Figure 2A–B, D–E). Probe appears as discrete, punctate signal at the cell periphery, and appears to move from the cell edges to the cell center in live imaging assays (Supplemental video 1 and 2).

Osteoclasts cultured on plastic show a 2-fold upregulation in cathepsin K gene expression compared to cells on dentin (Table 1). This upregulation is consistent with increased cathepsin K probe fluorescence in media from cells cultured on plastic rather than dentin (Figure 2G). Addition of cathepsin K inhibitor to cells reduces probe fluorescence in the media while maintaining cathepsin K mRNA expression in cells (Figure 2G, Table 1). This suggests a direct inhibition of cathepsin K enzyme on substrate cleavage without affecting gene expression.

Cathepsin K probe localizes adjacent to newly mineralizing bone in vivo

In vivo, CatK probe activation is apparent in regions neighboring newly mineralized bone. 7 day old neonatal mice were injected with both CatK probe and FRFP750- a near-infrared bone formation marker binding to mineralized surfaces [18] labeled in an optical wavelength distinct from the CatK probe. While FRFP binds to both actively forming and resorbing surfaces at a local level, when imaged macroscopically at the whole-bone or whole-animal level of resolution, pure osteolytic surfaces are not labeled in large enough quantity to be observed [18]. Thus, when imaging bone specimens or animals at low resolution, FRFP signal is highly reflective of newly mineralizing bone surfaces [18]. Planar fluorescence reflectance imaging (FRI) of dissected limbs reveals activation of CatK probe in the ulnar and radial distal growth plates neighboring regions of new bone mineralization (Figure 3A). Signal evident in the paw is due in part to autofluorescence from the skin which has been removed along the ulnar-radial length, but left intact on the paw. Both CatK (3B, left) and FRFP750 (Figure 3B, middle) show fluorescence along the length of the tail. Merging the two distinct channels and plotting fluorescence along the tail length reveals a staggered offset pattern reminiscent of regions of staggered formation and resorption typical in growth plate development (Figure 3C) [20].

Cathepsin K probe upregulation precedes bone loss in Ovariectomy

To monitor in vivo regulation of upregulated osteoclast activity, 10 week female Balb/c mice were subjected to ovariectomy (OVX), sham, or OVX+daily pamidronate (OVX+Pam) and assessed for CatK probe fluorescence and tibial bone loss on days 8 and 14. Bone loss due to

estrogen withdrawal is observed by micro-computed tomography (μ CT) in the proximal tibia by day 14, and is inhibited by daily pamidronate injections (Figure 4). CatK probe detects upregulated OVX-induced osteoclast activity before evidence of bone loss by μ CT (Figure 5). At 8 days post-surgery, OVX tibiae demonstrate significant increases in CatK fluorescence vs. OVX+Pam and sham controls. Fourteen days after OVX, CatK signal has returned to control levels (Figure 5). A subset of mice were dual injected with FRFP750 to monitor bone resorption and formation in parallel. 8 days post-OVX, near-infrared markers of bone formation and bone resorption demonstrate increased bone turnover in OVX compared to sham and OVX+Pam. At day 14, while resorption has returned to normal in OVX, formation remains elevated, demonstrating the characteristic behavior of bone formation lagging behind bone resorption.

Ex Vivo Specimen Imaging Reveals Spatially Uncoupled Activity

To assess spatial variation in bone formation and resorption, femora from mice dual-labeled with CatK and FRFP750 were dissected and imaged by planar fluorescence reflectance imaging. Fluorescence separation (FlSp), indicating the distance between peak fluorescence of FRFP750 and CatK probe was determined to demonstrate local spatial separation between bone formation and resorption (Figure 6B). During short-term bone loss, FlSp increases, suggesting greater spatial separation between formation and resorption as a result of OVX (Figure 6C). This increase in distance is fully prevented by daily Pam treatment, and returns to normal 14 days post-OVX, indicating a return to co-localization between formation and resorption in the growth plate.

Histology

Fluorescence microscopy of cathepsin K probe reveals punctate signal associated with the surface of trabecular bone as well as dispersed throughout the neighboring marrow (Figure 7A). This pattern is consistent with immunohistochemical staining for cathepsin K on neighboring sections (Figure 7B). Cathepsin K signal on the bone may reflect actively resorbing osteoclasts while signal in the marrow may represent either probe cleaved by pre-osteoclasts who have upregulated their cathepsin K expression, or cleaved probe fragments that have been released by actively resorbing osteoclasts along with other bone degradation products.

DISCUSSION

The cysteine protease, cathepsin K, is a biomarker for monitoring osteoclast activity in vivo. Cathepsin K is expressed by osteoclasts and a small number of osteoclast precursors, but is absent in osteoblasts and osteocytes [3], making its expression in bone specific for the resorption phase of bone metabolism. In an acidic environment, cathepsin K is sufficient for complete degradation of type I collagen, without contribution from other proteases [2]. Recently, cathepsin L and other as yet unidentified cysteine proteases have been implicated in cathepsin K-deficient calvarial bone resorption, while MMP's may compensate for cathepsin K-deficiency in long-bone resorption [21]. In vitro, the cathepsin K fluorescent probe demonstrates a 2-fold preferential activation by cathepsin K over cathepsins B and L, and a 14-fold preferential activation over MMP2 and MMP9 [17], suggesting that the cathepsin K probe demonstrates preferential selectivity towards cathepsin K over other proteases which may play a secondary role in osteoclastic bone resorption.

The CatK probe significantly increases its fluorescence due to cleavage of a cathepsin K-sensitive linker region following exposure to cathepsin K enzyme, incubation with osteoclasts in vitro, and after ovariectomy in mice prior to discovery of bone loss by μ CT. In vivo imaging reveals patterning of cathepsin K probe adjacent to newly mineralizing bone, consistent with cathepsin K activity observed in endochondral ossification, where osteoclasts are responsible for matrix turnover during development [20]. Following ovariectomy, spatial separation

increased between peak CatK probe signal and peak FRFP750 signal. We speculate that during normal remodeling at the distal femoral growth plate, osteoclast activity is spatially correlated with mineralization, but following estrogen withdrawal, osteoclast activity increases at sites proximal to the growth plate (Figure 6E). This increase in signal may represent removal of trabecular bone during the two weeks following OVX.

In the present study, cathepsin K probe was elevated at 8 days following OVX and returned to sham values by 14 days. This preceded significant trabecular bone loss, which wasn't observed until 14 days. Other investigators have shown similar transient increases in osteoclast activity followed by stable decreases in bone volume subsequent to ovariectomy. Blanque et al have shown a transient increase in pyridinoline/creatinine ratios peaking 2 to 5 weeks post surgery, depending on mouse genotype, returning to sham values thereafter [22,23]. Marrow cells isolated from OVX mice show a transient increase in osteoclast potential 7 days following OVX, with return to control values by 30 days [24]. While bone volume fraction stabilizes to a minimum between 28 and 42 days post-OVX of 8 week ddy mice, osteoclast number and surface parameters return to sham values by this later time point [25]. Thus, while structural indices of bone loss may persist long after hormone withdrawal, osteoclast activity maximizes and returns to sham values in a transient manner. The specific timing of this peak is likely to depend on both mouse age and genotype, as both demonstrate significant effects on bone loss following OVX [26–28]. Similarly, phenotypic studies in cathepsin K knockout mice suggest that withdrawal of cathepsin K has its most severe effects during periods of rapid growth, such as at 2 months of age [29]. Findings from the present study suggest a transient change in cathepsin K upregulation following OVX which returns to baseline values by 14 days.

In vitro enzyme assays demonstrate sensitivity of the cathepsin K-linker region to cathepsin K cleavage, rather than cleavage at the backbone poly-lys component of the substrate. These experiments were performed in buffer at pH 6.0 based on previous studies [16,17]. Cathepsin K has an optimal activity at pH 5.5[30], and is secreted by the osteoclast in an acidic environment of pH ranging from 4.7 to 6.8 [31]. It remains possible that decreasing the pH in our enzyme assays would further enhance probe activation, but it is unlikely that this would enhance cleavage of the probe in sites other than the cathepsin-K linker region.

In vitro, CatK probe appears as bright, intracellular signal, and probe trafficking between cell periphery and interior is evident. The mechanism of cell uptake and activation of the CatK probe is unclear. Osteoclasts have four distinct membrane domains [32]. During normal resorption, degradation products are endocytosed through the ruffled border of the osteoclast and subsequently transcytosed to the apical surface of the basolateral membrane by microtubules, where they are cleared from the cell [33–35]. An endocytotic pathway separate from the uptake of degraded bone matrix may be responsible for CatK probe uptake. Negatively charged dextrans have been shown to be endocytosed through the osteoclastic basolateral membrane by a pathway distinct from that responsible for the removal of degraded bone material [12]. Markers of endocytosis from the basal plasma membrane of osteoclasts get delivered to the ruffled border, and have been subsequently found intracellularly, within the resorption lacuna itself, and attached to the underlying bone matrix [36]. We hypothesize, then, that the cathepsin K probe is endocytosed from the basolateral surface of the osteoclast where it is directed toward the ruffled border and released into, or neighboring the region of high cathepsin K activity. Activation of probe fluorescence occurs, and cleaved probe by-products are likely transported by microtubules along with other bone degradation products to the apical surface of the cell, where they are subsequently released into the extracellular environment.

RAW264.7 cells cultured on plastic were less susceptible to cathepsin K inhibitor than cells cultured on mineralized dentin. The cathepsin K inhibitor used in this study is cell permeable, suggesting that there may be difference in membrane permeability between cells cultured on

the two substrates. Osteoclasts cultured on mineralized materials demonstrate significant differences in cytoskeletal organization compared to cells on non-mineralized plastic or glass. While osteoclasts cultured on dentin reorganize to form thick, actin-rich sealing zones, osteoclasts on plastic have thin, podosomal belts at the cell periphery [11,37]. This cytoskeletal rearrangement may alter cell permeability in a manner which makes cells cultured on dentin more susceptible to permeation and action of the cathepsin K inhibitor than cells cultured on plastic.

Cathepsin K probe activation may not be limited to actively resorbing osteoclasts, however, as monocyte-derived macrophages, mononuclear osteoclast precursors, and mononuclear osteoclasts exhibit staged cathepsin K expression through development [38,39]. Indeed, in RAW264.7 cultures treated with RANKL, mononuclear pre-osteoclasts demonstrate positive cathepsin K probe signal. These cells may activate cathepsin K probe by secreting active cathepsin K enzyme into an extracellular environment rich in probe concentration, or through endocytosis of probe followed by subsequent intracellular probe activation. Recently, dendritic cells have also been shown to express cathepsin K mRNA, but at much lower levels than osteoclasts [40]. It is unknown whether expression patterns of cathepsin K by dendritic cells change with hormone withdrawal.

Techniques to directly localize osteoclast location and activity in vivo are limited. Radiolabeled bisphosphonates used in bone scans fail to differentiate purely osteolytic lesions [41]. Pamidronate labeled with far-red fluorescent molecules can be viewed histologically at sites of both osteoblast and osteoclast activity, but fail to provide sufficient signal to monitor osteolysis non-invasively [18]. Accumulation of low molecular weight fluorescent markers in resorption pits has been proposed as a means to detect osteoclast resorption in vitro, however this technique has not been applied in vivo [12]. A fluorescent copolymer conjugate has recently been developed which appears to bind preferentially to resorption surfaces based on differences in mineral crystallinity [42]. However, it remains unclear whether this marker can differentiate quiescent, but highly mineralized bone from active resorption sites. By directly reporting cathepsin K activity, we have demonstrated early upregulation of osteoclast activity prior to significant downstream effects of bone loss, suggesting a valuable technique for prognostic indicators of increased osteoclast activity. The ability to monitor early osteoclast upregulation prior to significant bone loss has tremendous potential for early diagnosis of bone destroying diseases. This information may provide clinicians with an improved tool for designing treatment options, as well as early feedback on the efficacy of a chosen protocol before waiting for downstream gain or loss of bone takes place.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Selective cathepsin K-mediated fluorescence probe activation in vitro. CatK fluorescence increases 5–6 fold following addition of cathepsin K enzyme, and is inhibited with the addition of the cathepsin K inhibitor. Isomeric control probe (d-control) remains unaffected by addition of protease. Trypsin fails to cleave CatK or d-control probes after 22 hours exposure, but successfully cleaves PS680, which contains L-lys-lys bonds in its backbone chain. These findings demonstrate selectivity of reporter probe linker sequence to cathepsin K cleavage and resistance of d-poly-lysine backbone to degradation. Data represent mean $\pm$ s.d. of three independent trials; * $p < 0.05$ vs. CatK680.

Figure 2.

Osteoclast-mediated activation of CatK fluorescent probe. RAW264.7 cells were cultured for 6 days on plastic or dentin in the presence of 50 ng/mL RANKL to promote osteoclast differentiation. On day 6, cells on plastic were incubated with 0.2 μ M cathepsin K probe (red) for 4–5 hours and the nuclear marker Syto24 (green) for 1 hour, then imaged by live confocal microscopy. Multinuclear osteoclasts demonstrate vivid, punctate intracellular cathepsin K probe activation (red) which appears perinuclear (green) (A, B). Increasing photomultiplier tube signal of confocal microscope reveals distribution of probe from cell periphery toward cell center in organized manner (D, E). Active trafficking of probe between periphery and cell body is apparent (Supplemental videos). (C,F) Osteoclast cultures reveal mixed morphology and altered actin distribution following fixation, permeabilization, and staining for TRAP (blue intracellular spots) F-actin (red), and nuclei (green). G) Media taken from osteoclasts demonstrates significant reduction in probe fluorescence when incubated in the presence of cathepsin K inhibitor. Media from cells on plastic show higher probe activation than cells on dentin, consistent with cellular expression of cathepsin K mRNA (Table 1). Two-fold upregulation of cathepsin K mRNA from cells cultured on plastic correlates with increased cathepsin K probe activation. Addition of cathepsin K inhibitor fails to significantly alter cathepsin K gene expression, indicating fluorescence change seen in G results from inhibitor effects on protein, not genomic reductions in cathepsin K expression. Values represent range of mean $\pm$ SD. Scalebars represent 10 μ m. *p<0.05 vs. 0 inhibitor

Figure 3.

Imaging of bone development in the neonatal mouse in vivo. A d7 mouse was labeled with CatK probe for bone resorption and FRFP750 to denote regions of newly forming bone. A) Left foreleg and paw imaged in white light (WL), CatK680, and FRFP750 demonstrate offset patterns of cathepsin K activation and mineralization in the ulna and radius. Skin present on paw demonstrates significant autofluorescence rather than increased probe activity. B) Tail images showing CatK680, FRFP750, and merged fluorescence. Line in merged image refers to C) plot profile of fluorescence of CatK680 and FRFP750, suggesting spatially staggered bone formation and resorption at developing tail vertebral growth plates. Color bars represent fluorescent intensity from low to high.

Figure 4. 10 week female Balb/c mice were subjected to OVX, sham, or OVX+daily pamidronate (Pam) injections. MicroCT from proximal tibiae reveals significant loss of bone in OVX animals by day 14. * p<0.05 vs. sham; ** p<0.05 vs. OVX-PAM.

Figure 5.

In vivo simultaneous imaging of bone resorption and bone formation in two distinct optical wavelengths. Mice were subjected to OVX and imaged for cathepsin K probe activation 8 or 14 days post OVX. A) White light image of mouse in FMT scanner showing 10 mm tibial region of interest. B) CatK fluorescent signal (yellow) with 10 mm masked tibial region of interest highlighted (rainbow) shows variable intensity at tibial region of interest. C) In 8 day short term bone loss, ovariectomy significantly increases CatK fluorescence over sham, OVX + Pamidronate, and OVX+d-control probe (white bar). Increase in resorption is coupled to increased formation (FRFP750, D). By 14 days post-OVX, resorption has normalized and returned to sham levels (E,F). * $p < 0.05$, ** $p < 0.001$ vs. OVX-CatK probe.

Figure 6.

Spatial discrimination of bone resorption and formation in the distal femur. Femora from OVX mice were dissected and imaged via planar fluorescence reflectance imaging (A). Scalebar shows probe fluorescence for FRFP750 and CatK signal scaled to maximum values for each specimen (B) Transverse fluorescence was plotted along the femoral length from the lateral third trochanter (arrow, A) to the distal femoral condyles. Location of peak transverse fluorescence was noted and the distance separating peak cathepsin K and peak FRFP750 signal was found. (C) 8 days post-OVX, cathepsin K probe signal becomes spatially uncoupled from sham and OVX+Pam, and returns to normal by 14 days (D). (E) Schematic representation of uncoupled bone resorption and formation in the distal femur following OVX. In non-OVX conditions, resorption closely follows formation in the growth plate region. Following OVX, the maximal resorption signal (CatK680, red) occurs proximal to the growth plate (arrow) at the site of existing trabeculae. In contrast, the region of maximum formation (FRFP75-, yellow) was not affected by OVX.

Figure 7. Non-decalcified cryosections of trabecular bone within marrow space demonstrates punctate cathepsin K probe staining in the marrow space and adjacent to the trabecular bone (A arrows), while cathepsin K immunostaining reveals punctate staining at comparable locations (B arrows). 20X magnification, scalebar = 50 μ m.

Table 1

Osteoclasts were cultured on dentin or plastic and assessed for change in cathepsin K mRNA between surfaces. A two-fold upregulation of cathepsin K mRNA from cells cultured on plastic vs. dentin correlates with increased cathepsin K probe activation between these surfaces (Fig 2E). Addition of cathepsin K inhibitor fails to significantly alter cathepsin K gene expression on both plastic and dentin, indicating fluorescence change seen in Fig 2E results from inhibitor effects on protein, not genomic reductions in cathepsin K expression. Values represent range of mean $\pm$ SD

Effect	Fold up-regulation of CatK mRNA
Cells cultured on plastic vs. dentin- no CatK inhibitor	2.18 (1.31–3.64)
Addition of 10 μ M CatK inhibitor to cells on plastic	1.23 (0.83–1.82)
Addition of 10 μ M CatK inhibitor to cells on dentin	0.72 (0.50–1.04)