Published in final edited form as:

Calcif Tissue Int. 2009 March ; 84(3): 229–239. doi:10.1007/s00223-008-9214-6.

Ablation of Cathepsin K Activity in the Young Mouse Causes Hypermineralization of Long Bone and Growth Plates

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Abstract

Cathepsin K deficiency in humans causes pycnodysostosis, which is characterized by dwarfism and osteosclerosis. Earlier studies of 10-week-old male cathepsin K-deficient (knockout, KO) mice showed their bones were mechanically more brittle, while histomorphometry showed that both osteoclasts and osteoblasts had impaired activity relative to the wildtype (WT). Here, we report detailed mineral and matrix analyses of the tibia of these animals based on Fourier Transform Infrared (FT-IR) microspectroscopy and imaging. At 10 wks, there was significant hyper-calcification of the calcified cartilage and cortices in the KO. Carbonate content was elevated in the KO calcified cartilage, cortical and cancellous bone areas. These data suggest that cathepsin K does not affect mineral deposition but has a significant effect on mineralized tissue remodeling. Since growth plate abnormalities were extensive despite reported low levels of cathepsin K expression in the calcified cartilage, we used a differentiating chick-limb bud mesenchymal cell system that mimics endochondral ossification but does not contain osteoclasts to show that cathepsin K inhibition during initial stages of mineral deposition retards the mineralization process while general inhibition of cathepsins can increase mineralization. These data suggest that the hypercalcification of the cathepsin K-deficient growth plate is due to persistence of calcified cartilage and point to a role of cathepsin K in bone tissue development as well as skeletal remodeling.

Keywords

Bone Turnover; Remodeling; Bone and Cartilage Development; Bone Architecture/Structure; cathepsin K; knockout mouse; FTIR spectroscopic imaging; growth plate; mineralization

Introduction

Cathepsin K is a cysteine protease with collagenolytic, elastinolytic and proteoglycan degradative activities known to be involved in early osteoclast action [1,2]. The enzyme is expressed by osteoclasts, chondroclasts, osteoblasts, hypertrophic chondrocytes [3,4], and other cells including endothelial cells [5], gastric mucosa [6], and skeletal and non-skeletal tumors [7,8]. Its highest level of expression is in osteoclasts [1]. Cathepsin K is also thought to be involved in proteoglycan modification in cartilage based on its ability to cleave link protein and aggrecan [9], although observations in articular cartilage suggest its role is via

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activation of pre-metalloproteases or through modifying interactions of glycosaminoglycan fragments with collagen [9].

Patients lacking cathepsin K activity due to genetic mutations have a form of skeletal dysplasia known as pycnodysostosis that is characterized by osteosclerosis, short stature, and bone hypoplasia [10] suggesting a significant role for the enzyme in endochondral bone growth. Material properties of iliac crest biopsies from two patients with pycnodysostosis (a 5-year-old boy and a 20-yr-old woman) compared to healthy control data showed the abnormal persistence of calcified cartilage in the cancellous bone matrix, disordered lamellar bone, and thickened mineral crystals [11].

The purpose of this study was to further characterize the bone material properties in an animal model of pycnodysostosis [12], the cathepsin K knockout (KO) mouse, as contrasted with age-, sex- and background- matched wildtype (WT) and heterozygous (HET) mice. The effects of ablating the cathepsin K gene in mice were first described by Saftig et al. [13] and Gowen et al. [14]. The phenotype of these animals mimicked the pycnodysostosis phenotype with mice lacking cathepsin K having thicker bones and more numerous trabeculae and increased numbers of osteoclasts relative to age-matched WT controls. The KO mice also had an accumulation of collagen in their osteoclasts and other cells [15]. A recent study characterized the histology and mechanical properties of 10-week-old KO mice with a C57Bl6/J background [16]. The bones of the cathepsin K-deficient B6 mice had mild osteosclerosis in areas of cancellous bone, with histomorphometrically defined increased trabecular bone mass and 2-fold greater trabecular numbers. The cortical bone area was also increased, with diaphyseal thickening with regions of disorganized lamellae. Diaphyseal strength was increased in the males, but not in the females, while there was a decrease in work to failure and post-yield deflection (i.e., bones were more brittle) in both sexes [16]. Mechanical parameters in the heterozygotes were intermediate. The present study quantifies the mineral properties in the tibia from the male KO animals from that same study contrasted with male WT controls and (HET) litter mates, and further tests the hypothesis that cathepsin K inhibition alters mineral properties of calcified cartilage during endochondral ossification by preventing degradation of proteoglycan inhibitors of mineralization.

Materials and Methods

Bones

The cathepsin K KO mice were developed in a C57Bl6/J background as reported elsewhere [15]. The left tibias from that study of 10 wk old male mice were used in the present study for histology to measure growth plate thicknesses and for FT-IR measurements. Only males were analyzed because prior analysis of mechanical properties failed to show abnormalities in female KO mice. There are other reports of sexual dimorphism in bone properties which were reviewed elsewhere [17] and osteoclast activation during puberty has been linked to expression of the androgen receptor [18], perhaps accounting for the greater differences in the young males as opposed to females.

Proximal halves of tibiae from 5 KO, 6 WT and 4 HET mice were immersion fixed in 10% neutral buffered formalin. A power study based on FTIR imaging of other 10 [19] and 12 [20] week old mice indicated $n=5$ would be sufficient to show significant differences ($\alpha = \beta = 0.05$), however bones from only 4 HET mice were available. After immersion fixation the bones were dehydrated with ethylene glycol monoethyl ether (Fisher, Fair Lawn, NJ), cleared in methyl salicylate (J. T. Baker, Phillipsburg, NJ) and embedded in methyl methacrylate with 15% dibutyl phthalate (Fisher Scientific). Undecalcified frontal sections were cut using a Leica 2050 microtome (Leica, Nussloch, Germany) from the equivalent depth within each sample. Sections for histological staining (5 μ m thick) sections were adhered to

charged slides, deplasticized and stained using Toluidine Blue to highlight the growth plate cartilage. Growth plate widths were measured under brightfield microscopy using an OsteoMeasure system (Osteometrics Inc, Atlanta, GA) connected to a Zeiss Axioskop microscope. For each section the epiphyseal and metaphyseal boundaries (defined by the last intact hypertrophic chondrocyte) of the entire growth plate were traced and the mean cord length between these boundaries computed; two sections per animal were measured. A single observer who was blinded to the specimen identity made all measurements. Mean $\pm$ SD for each genotype was then calculated.

FTIR Microscopy and Imaging

Two μ m thick unstained sections were used for FT-IR microscopy analysis; these were placed on barium fluoride windows (Spectra Tech, Hopewell Junction, NY). A minimum of 5 sections from each animal was first used for point-by-point analysis (pixel resolution $25 \mu\text{m} \times 25 \mu\text{m}$), and then a minimum of 3 sections from each animal were used for higher resolution FT-IR imaging spectroscopy (FTIRI) to achieve a pixel resolution of $6.25 \mu\text{m} \times 6.25 \mu\text{m}$. Light microscopic images were used to select areas that contained only calcified cartilage, areas of cortical bone in the midshaft, or areas of cancellous bone in the metaphysis. For the point-by-point analyses 10 spots in each type of bone were analyzed. For the imaging data areas corresponding to each bone type were defined. All data were collected on a Perkin-Elmer 300 Spotlight infrared imaging system (Perkin Elmer Instruments, Shelton, CT, USA). Background spectra were collected under identical conditions from the same BaF₂ windows. After acquisition, spectra were truncated to allow analysis of the regions of interest, zero-corrected for baseline and the spectral contribution of PMMA embedding media subtracted using ISYS Chemical Imaging software (v 2.1, Spectral Dimensions Inc., Olney, MD, USA). The point-by-point data gives better signal to noise, but does not allow mapping of images of the areas being examined, thus both techniques were used and parameters derived in each examined separately. For both analyses, the spectral resolution was 8 cm^{-1} . For FTIR imaging (FTIRI), spectra were transferred to yield images corresponding to infrared band areas, peak height ratios and integrated area ratios by a combination of instrument software and ISYS Chemical Imaging software.

The spectroscopic parameters calculated for both point-by-point and imaging data were: mineral/matrix ratio, carbonate/phosphate ratio, crystallinity, and collagen cross link ratio (XLR). The mineral/matrix ($\nu_1, \nu_3 \text{ PO}_4$ band $[900\text{--}1200 \text{ cm}^{-1}]$ /Amide I band $[1590\text{--}1720 \text{ cm}^{-1}]$ integrated areas ratio) corresponds to ash-weight measurements [21]. Carbonate/phosphate ratio was calculated from the integrated areas of the carbonate band at $850\text{--}890 \text{ cm}^{-1}$ /phosphate area, and mineral crystallinity was calculated from the intensity ratios of subbands at 1030 (stoichiometric apatite) and 1020 cm^{-1} (non-stoichiometric apatite). Carbonate/amide I (carbonate/matrix) ratio [22] was calculated to eliminate the effect of changes in phosphate on the assessment of carbonate incorporation into mineral. XLR, a parameter reflecting the relative ratio of non-reducible and reducible collagen cross-links, was expressed as the intensity ratio at two specific wavelengths (1660 and 1690 cm^{-1}). Details for the spectral processing methods and reproducibility of measurements are reviewed elsewhere [23]. In the spectral images, pixels devoid of bone (no mineral and/or matrix spectral signature) were set equal to zero and masked to be excluded from calculations. The imaging results were expressed as histograms describing the pixel distribution of the parameters above, mean values and standard deviations of the pixel distributions, and representative color-coded images. These means and standard deviations were averaged for multiple sites in each animal and among the different animals for each age and genotype using Microsoft EXCEL. Data were analyzed by analysis of variance (ANOVA). Post-hoc comparisons were made using Tukey pairwise mean comparison tests. Analyses were conducted using InStat (GraphPad InStat, v 2.0, Carlsbad, CA).

Differentiating Chick Limb-bud Cultures

Fertilized eggs were incubated for four and a half days in a Napco circulated air incubator. After incubation, stage 21-23 limb buds [24] were removed from the chick embryos under a microscope and trypsinized in 1X Trypsin-EDTA solution in a water bath at 37 °C for 10 min to liberate the mesenchymal stem cells. The suspension was filtered through a double layer of 20µm Nitex membrane to remove any contaminants. Cells were counted on a hemocytometer, checked for viability (trypan blue dye exclusion), and pelleted at 1,000 rpm for 8 min at 4 °C. Cells, resuspended in DMEM (Dulbecco's Modified Eagle Medium) containing 1.3 mM CaCl₂, 25 µg/ml freshly prepared sodium ascorbate, 0.3 mg/mL L-glutamine, antibiotics and 10% fetal bovine serum were plated as micromass spots at concentrations of 0.75-1.0×10⁶ per 20 µL in 35×10 mm culture dishes. Cells were allowed to attach for 2 h after which they were flooded with 2 mL media. Cultures were maintained in a CO₂ incubator at 37 °C and 5% CO₂. From day 2 onward, the inorganic phosphate (Pi) content of the “mineralizing” cultures was adjusted to 4 mM, while control, non-mineralizing cultures, were maintained with 1 mM Pi. Media was changed every other day.

Two cathepsin inhibitors were used: 0-2 uM cathepsin K inhibitor II (Calbiochem), a “specific” cathepsin K inhibitor (Boc-phe-leu-NHNH-CO-NHNH-Leu-Z) and 0-2 uM cathepsin inhibitor III (Calbiochem) an inhibitor of cathepsins B/L/S (Z-Phe-gly-NHO-Bz-pOMe). Cathepsin K inhibitor II was reported at similar concentrations to completely block cathepsin K activity in human synovial fibroblast cultures [25] and cartilage [26] without significantly inhibiting cathepsin L [25], while cathepsin inhibitor III inhibits cathepsin B, L, and S, and papain but not cathepsin K [27,28]. After pilot studies to insure that the inhibitor doses used were not toxic (based on comparative temporal changes in DNA content [29] and the mitochondrial tetrazolium salt (MTT) assay (ATCC, Manassas, VA) of control and inhibited cultures), the cathepsin K specific inhibitor (cathepsin inhibitor II) was used in all studies at a final concentration of 1.43uM and the cathepsin inhibitor III at a final concentration of 2.5uM. These inhibitors or the carrier medium was added to the cultures from day 5, 7, 9 or day 11/12, corresponding to times immediately before, or immediately after the start of mineral deposition. ⁴⁵Ca at a concentration of 0.5 µCi/mL was added to the culture dishes on day 7 and with every medium change thereafter to monitor mineral accumulation. At the indicated time points, micromass cultures were washed in phosphate-buffered saline (PBS), lifted from the dish, and transferred to scintillation vials. Culture dishes were then flushed twice with 200 µL of 4N hydrochloric acid to dissolve any remaining mineral. The hydrochloric acid extract was then added to the scintillation vials. To solubilize the mineral in the cultures, vials were sealed and placed in a 60 °C oven for one hour and then cooled. Aquasol (5 mL) was then added to each vial and vortexed until the liquid was free of any cloudiness. Scintillation counting was performed on a Beckman scintillation counter. The amount of ⁴⁵Ca uptake was corrected for uptake in similarly treated non-mineralizing controls and then normalized to the uptake at day 21 in untreated mineralizing control (4P) cultures. All results were expressed as mean ±SD for a minimum of three separate experiments at each time point.

At selected time points, cultures were also sampled for histochemical visualization of calcium phosphate mineral using the phosphate-specific von Kossa technique or alizarin red staining for calcium as detailed elsewhere [30]. To localize cathepsin K protein, cultures were fixed for 4 hours with formaldehyde freshly prepared from 4% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.4. Cultures were then removed from the dish and processed for paraffin embedding and sectioning. Five micron thick sections were prepared for immunostaining by blocking endogenous peroxidase (2% hydrogen peroxide in PBS) and then subjected to protein block before adding 250ng of antibody (MAB3324, Millipore Company, Cathepsin K monoclonal) to each slide. Negative controls received no antibody, but were handled similarly. A section of a rat growth plate was used as a positive control. The slides were maintained at 4°C in a

closed moist chamber, overnight, then rinsed with PBS, and subjected to streptavidin/biotin secondary antibodies, conjugated to peroxidase. A brown reaction product was produced when the sections were treated with diaminobenzidine in the presence of hydrogen peroxide. A brief hematoxylin stain was used for counterstaining.

Results

In this study, we investigated the material properties of bones from cathepsin K KO as well as WT and HET controls based on Fourier transform infrared micro-spectroscopy and spectroscopic imaging. We then probed the mechanism of the previously unreported alterations in the cathepsin K KO growth plates using avian cell culture studies.

A comparison of the histology of the WT, HET, and KO mouse tibias, focusing on the proximal epiphysis (Figure 1a,b) showed that the growth plate was significantly wider in the KO than that in the WT ($127 \pm 14 \mu\text{m}$ vs. $108 \pm 13 \mu\text{m}$, $p < 0.001$); growth plates in HET bones had a variable intermediate thickness. The average thickness of the growth plate from the top of the resting zone to the bottom-most hypertrophic cell is plotted in Figure 1c.

Characterization of Bone Material Properties using Infrared Spectroscopy

FT-IR spectroscopic imaging showed that the mineral/matrix ratio in the calcified cartilage below the epiphysis was elevated in the KO relative to the HET and WT (Figure 2a). This increase can be seen in the individual FT-IR images comparing WT and KO growth plates (Figure 2a), in the averaged data from all FT-IR images (Figure 3), and in Table 1 which summarizes all data from the point-by-point analyses. The average values for the point-by-point data for the growth plate calcified cartilage mineral/matrix ratios were different from values in the higher resolution FTIRI for both KO and WT, although as in the images, the KO had significantly higher mean mineral/matrix than the WT. The only other point-by-point FTIR parameter that was significantly different when the average growth plate calcified cartilage data was compared with the imaging data was XLR. With the exception of mineral/matrix ratio, none of the other point-by-point parameters were significantly altered in the calcified cartilage (Table 1). In contrast, the mineral/matrix and carbonate/phosphate values were significantly increased in the averaged high resolution FTIR image data (Figure 3), while crystallinity (Fig. 2b) was slightly but not significantly decreased. The pixel distribution for the crystallinity parameter in the KO was more uniform than that of the WT (Figure 2b). Carbonate/amide I ratio was significantly greater in the KO (0.036 v 0.027 , $p = 0.04$) indicating the value of the carbonate/phosphate ratio was in part diminished because of the elevated mineral content of the KO growth plate. FTIRI data is not presented for HETs because sample size was too small and data heterogeneity was too broad to reach statistical significance for either the point-by-point data (Table 1) or the images (not shown).

In tibial cortices of the KO, the mineral/matrix ratio was increased compared to WT based on point-by-point analysis (Table 1) and average FTIRI (Figure 3) and individual FTIRI images (Figure 4). Based on FTIRI, the collagen cross link ratio, XLR, was not significantly altered in the cortical or cancellous bone areas of the KO (Table 1, Figure 3, Figure 4). The carbonate/phosphate ratio was elevated in the both the KO cancellous and cortical bone areas (Figure 3, Figure 4, Table 1), remaining significantly elevated in both cortical and trabecular bone when expressed as carbonate/amide I (0.04 vs 0.02 (cancellous, $p = 0.01$); (0.05 vs 0.04 (cortical, $p = 0.04$)). Crystallinity was not significantly altered in the midshaft cortices of the KO relative to the WT images, or in the KO cancellous bone in the metaphysis. There was a slight but significant decrease in the cortical crystallinity in the point-by-point images. In general, the pixel histograms for the FTIR images showed a more uniform distribution of all parameters in the KO compared to the WT (data not shown).

Effects of cathepsin inhibition in differentiating mesenchymal cell cultures

To distinguish between a direct effect of impaired osteoclast activity in the growth plate, and the need for cathepsin K to modify the proteoglycans within the mineralizing matrix we determined the effect of inhibiting cathepsin activities on mineral accretion in the osteoclast-free chick limb-bud micromass culture system [30,31,32]. In these cultures, prior to the start of mineralization, there is abundant production of large aggregating proteoglycans [33,31]. As in the rodent growth plate (Figure 5a-A) Cathepsin K protein persists in the culture (Figure 5a-B) around the chondrocyte nodules and in the cells throughout the course of the culture period as seen at day 21. The negative control (figure 5a-C) has no brown staining. When a “specific” cathepsin K inhibitor was added before (day 7) or just after (day 9) initial mineral deposition, mineral accrual was retarded (Figure 5b). When that inhibitor was added after mineral proliferation had begun (days 11, 14 or 16), no significant effect was noted. Similarly, use of a broad spectrum cathepsin B/S/L inhibitor resulted in decreased mineral accretion when added early (day 9; Fig. 5c) but caused enhanced mineral accretion when added at day 12 or 14 (Figure 5c).

Discussion

This study using FTIR measurements has demonstrated that material properties (mineral content and mineral composition), in addition to the previously demonstrated whole bone mechanical and geometric properties in young mice lacking cathepsin K, are distinct from those in age- and sex- matched wildtype animals. These findings are in agreement with the data reported from a study of a young child with pycnodysostosis [11], and provide further explanation of some of the mechanical properties (i.e., reduced fracture toughness) previously reported in these mice [16]. In addition to confirming the report of excessive calcified cartilage retention in the cancellous bone areas of humans with defects in this enzyme, this study demonstrated that the growth plate of cathepsin-K deficient animals was excessively mineralized, suggesting that cathepsin K may be involved in the removal of calcified cartilage during endochondral ossification as the extent of calcified cartilage retention in the trabecular bone varies inversely with the extent of remodeling. This suggestion was verified by cell culture studies in an avian system. The results of these studies indicate a role for cathepsin K during growth and development of bone as an organ in addition to its well known role in the remodeling of the bone tissue.

There were four consistent findings of this study. First, mineral content of the KO calcified epiphyseal cartilage and the cortices were significantly increased relative to WT. Second, in contrast to this, mineral content in the KO metaphyseal cancellous bone was not significantly changed. Third, crystal size and perfection were decreased in the KO calcified cartilage while carbonate content was increased. Lastly, the observations in chicken limb-bud micromass cultures where cathepsin activities were inhibited do not mimic the findings in the KO mouse. This discrepancy between the KO and the mineralizing cultures argues that the matrix defects in the KO mice bones are due to impaired remodeling rather than direct effects on chondrocyte-mediated mineralization.

Based on the culture data, the KO growth plate observations, can be attributed to the failure to remodel calcified cartilage. Even in the WT, the growth plate mineral/matrix ratio exceeds that in the cancellous bone of the metaphysis. Similar persistence of calcified cartilage, with decreases in crystallinity and increases in mineral/matrix ratio, but without changes in collagen maturity was found in an infant with a different form of osteopetrosis [34], and decreased crystallinity was noted many years ago in x-ray diffraction analyses of a rodent model of osteopetrosis [35]. As reviewed elsewhere [36], in these cases, and in other examples of osteopetrosis not associated with cathepsin K deficiency [37,38], the growth plates similarly had increased mineral content, and calcified cartilage bars persisted in the metaphyseal bone.

While it is likely that defective osteoclastic activity is the only cause of the excessive growth plate calcification, it is possible that in the absence of cathepsin K expression by hypertrophic chondrocytes [2,39], the mineralization process in the KO might be different from that in the WT.

The lack of change in the mineral/matrix ratio of the metaphyseal bone of the KO as compared to the WT despite the significant increase in carbonate/phosphate ratio and carbonate/amide I ratio in the KO, is also most likely due to the persistence of the calcified cartilage bars in the KO metaphysis. Since the KO calcified cartilage has a higher mineral/matrix ratio than the KO bone, an increase in the proportion of calcified cartilage within the metaphysis could result in a broader distribution of mineral/matrix values, and the failure to detect significant changes such as those seen in the KO cortical bone. The increased carbonate incorporation into the hydroxyapatite crystals as indicated by both the carbonate/phosphate ratio and the carbonate/amide I ratio shows that more older calcified tissue is present, as carbonate incorporation into bone mineral increases with age [40], relating these changes to impaired remodeling.

Other than the small but significant changes in carbonate incorporation and the decreased crystallinity in the KO epiphyses, there were no consistent compositional changes in the mineral. A significant decrease in crystal size/perfection was noted in the lower resolution point-by-point data, most likely because of the higher signal/noise, but with the number of samples available the decrease was not significant in the FTIRI data. The smaller crystals are likely related to the defective remodeling along with the continued formation of new calcified tissue. The hypermineralization, the decreased crystal size, and the slight but not consistent increased matrix maturity all could contribute to the previously reported [16] impaired mechanical performance of the KO bones.

The lack of agreement between the average numerical data for imaging and point-by-point parameters in the calcified cartilage reflects the greater spatial variation in this region, such that by sampling 25 $\mu\text{m} \times 25 \mu\text{m}$ areas (point-by-point) too large a region was evaluated, while there was discrete spatial variation noted at 6.3 $\mu\text{m} \times 6.3 \mu\text{m}$. This lack of agreement between imaging and point-by-point data was not seen in the less heterogeneous metaphysis and cortices.

The avian cell culture data confirmed the suggestion that the hyper mineralization of the growth plate was due to the persistence of calcified cartilage. The chick limb-bud mesenchymal cell culture system used mimics many of the morphologic and biochemical changes seen in the vertebrate growth plate, but lacks osteoclasts. The culture system has been used to investigate individual processes involved in both chondrocyte differentiation [41] and cartilage calcification [30-33]. Prior studies demonstrated reduction of proteoglycan size either by altered synthesis or enzymatic degradation enhanced calcification in this culture system [31]. The cathepsin K activity in the avian is analogous to that in vertebrates since chick Cathepsin K activity can be used to monitor human cathepsin K activity [42], and the comparable distributions of this activity in the rodent growth plates and chick cultures suggest that the chick and vertebrate enzymes are similar. The cathepsins, in addition to matrix metalloproteases (MMPs), appear from the cell culture data to also be involved in the degradation of proteoglycans in the growth plate, as the ^{45}Ca uptake in the mineralizing cultures in the presence of both the cathepsin inhibitors tested was identical to that in the control (non-mineralizing) cultures, and did not show the uptake found in mineralizing cultures without cathepsin inhibitors. Growth plate proteoglycans are inhibitors of hydroxyapatite formation in vitro and in vivo [43]; hence their removal should facilitate initial mineral deposition. This is in agreement with the finding that the most dramatic decrease in mineral accumulation with both cathepsin inhibitors occurred in the chick limb-bud cultures when the inhibitors were added just as mineralization commenced, i.e., from day 9 onward. In contrast, there was an

increase in mineral accretion when the inhibitors were added after mineral deposition had already started.

These in vitro data indicate that impaired removal of the calcified cartilage in the mouse is a more likely explanation than a direct effect of chondrocyte cathepsin K on mineralization. Based on the avian culture data where early cathepsin K inhibition retarded mineral accumulation, one would expect that were the defect due to the absence of cathepsin K production in hypertrophic chondrocytes of the mouse, there would also be impaired calcified cartilage formation rather than hyper mineralization. Accumulation of smaller proteoglycans, such as decorin and biglycan in the cathepsin K KO could enhance mineral formation [44]. Persistence of proteoglycans in the mouse may also lead to calcium chelation, and hence enhanced mineralization [45]. The presence of other enzymes that can modify the proteoglycans in the cathepsin K-null mouse [46] may also explain why the inhibition of mineralization is not seen in the cathepsin K KO. It seems more likely that cartilage calcification is not impaired in the KO mice, but rather that there is a persistence of the calcified cartilage because the absence of properly functioning osteoclasts (osteoclasts lacking cathepsin K) prevents calcified cartilage from being effectively remodeled. This would imply that the crystal sizes in the calcified cartilage would be decreased, which was suggested by the FTIR imaging data from the growth plate, because the smaller crystals are generally removed first, but where this cannot occur, while some crystals continue to grow, the average sizes would tend to be lower.

A limitation of this study was that the mineral properties were characterized in animals of a single age, single sex, and that there were few HET mice available. While the variability (large SD) in the HET animals may reflect the smaller sample size, this type of variability was noted when reproducibility in a single HET bone was contrasted to reproducibility in either the WT or the KO. The most likely reason for this variation is different extents of penetrance within the cells in the HET animals as well as among animals. We have previously noticed this phenomenon in other mouse models [47].

In summary, the current studies provide the first report of changes in composition and other material properties in the cathepsin K KO mouse. The results suggest that cathepsin K does not directly affect mineral deposition. Rather, it has a significant effect on mineralized tissue remodeling. The absence of cathepsin K led to hypercalcification of the growth plate and altered cortical bone composition, due principally to the persistence of calcified cartilage, and suggests that failures to appropriately remodel the growing tissue in the absence of this enzyme may underlie the poor tissue mechanical quality and increased bone fragility reported in association with cathepsin K deficiency.

Acknowledgements

This study was supported by NIH grants AR037661, AR046121 and AR41210.

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Figure 1.

Histology of the growth plate in WT, HET, and KO mouse tibias. a) Typical sections from WT and b) KO growth plates. Original magnification 20X; scale bar = 100 μm. c) Growth plate widths within the epiphyseal and metaphyseal boundaries (defined by the last intact hypertrophic chondrocyte) expressed as mean $\pm$ SD. * $p < 0.05$ relative to WT.

Figure 2.

FTIR Images of the growth plate in cathepsin K-KO and WT tibias: a) mineral/matrix ratio and b) crystal size and perfection (crystallinity) in the same sections. The color bars represent the scales for each of the parameters. The axes are in pixels, where 1 pixel ~6.3 μ m. The adjacent pixel histograms show the distribution of pixels with each value in the corresponding image. The pixel histograms were expanded to be of comparable value distributions to facilitate visual comparison.

Figure 3.

Mean and standard deviation of FTIR imaging analysis of bone sections from growth plate (GP), metaphyseal cancellous bone (TB), and midshaft cortical bone (CB). Data is shown for WT (n=6) and KO (n=5). Parameters shown are: M/M (mineral/matrix ratio), XLR (collagen cross-link ratio), XST (crystallinity = crystal size/perfection), and carbonate/phosphate ratio (C/P). C/P multiplied by 1000. *p<0.05 relative to WT for same parameter.

Figure 4.

FTIR Images of midshaft cortical and metaphyseal cancellous bone in KO and WT tibias. Typical images from the same sample are shown for mineral/matrix ratio, carbonate/phosphate ratio, collagen maturity, and crystallinity. The images for each parameter in each bone type are based on the same color scale. In these images one pixel = 6.25 μm .

Figure 5.

a) Distribution of cathepsin-K in: (A) the rat growth plate, (B) a micromass cultures of differentiating chick limb-bud mesenchymal cells at day 21 in mineralizing media (4mM Pi), (C) a negative control of day 21 chick cells in mineralizing media without the antibody.

b) Kinetics of ^{45}Ca uptake in differentiating micromass cultures in the presence and absence of the specific cathepsin K inhibitor. The y axis shows the differential uptake (mineralizing-control) for each treatment condition, normalized to the uptake at day 21. Error bars are SD for three independent experiments, run at different times. The day at which addition of the cathepsin K specific inhibitor (1.4 μM) started is shown. The lines are the best fits to the mineralizing control data (heavy solid line), the data at day 9 (dashed line, overlays day 5 and 7 data), and the data at day 14 (dashed-dotted line; overlays day 11 data).

c) Mean differential ^{45}Ca uptake in micromass cultures in the presence and absence of the general cathepsin B/S/L inhibitor added from day 9 at day 16, 19, and 21 ($n=3$). * $p<0.05$ significantly different from control mineralizing cultures without the inhibitor.

Table 1
FTIR Parameters from Point-by-Point Analyses

	WT N=6	HET N=4	KO N=5
Growth Plate Calcified Cartilage			
Min/mat	5.48 ± 0.47 ^{**}	5.85 ± 1.5	6.32±0.55 ^{*,**}
CO3/P	0.0044±0.002	0.0067±0.005	0.0074±0.005
CO3/Am I	0.025±0.01	0.035±0.02	0.046±0.02
XST	1.13±0.05	1.12±0.008	1.11±0.06
XLR	5.35±0.46 ^{**}	5.56±0.72	6.56±1.06
Cancellous Bone in the Metaphysis			
Min/mat	5.78±0.89	5.94±1.0	6.22±0.96
CO3/P	0.0064±0.001	0.0069±0.0005	0.0074±0.005
CO3/Am I	0.0064±0.002	0.0070±0.0005	0.0095±0.002 [*]
XST	1.13±0.02	1.13±0.02	1.12±0.02
XLR	3.65±0.38	3.74±0.19	3.86±0.72
Midshaft Cortical Bone			
Min/mat	6.03±0.28	6.14±0.31	6.58±0.10 [*]
CO3/P	0.0074±0.003	0.0075±0.003	0.0088±0.002
CO3/Am I	0.0046±0.002	0.0045±0.003	0.0058±0.015
XST	1.13±0.01	1.15±0.01	1.09±0.01 [*]
XLR	3.39±0.05	3.57±0.08	3.59±0.13 [*]

* Significantly different from WT (p<0.05)

** Significantly different from same parameter in higher resolution FTIR images