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Biochemical and Biophysical Research Communications 332 (2005) 248–253
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Altered cathepsin D metabolism in *PHEX* antisense human osteoblast cells ☆

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Received 2 April 2005
Available online 30 April 2005

Abstract

X-linked hypophosphatemia (XLH), the most common form of hereditary rickets, is caused by loss-of-function mutations of *PHEX* gene in osteoblast cells, leading to rachitic bone disease and hypophosphatemia. Available evidence today indicates that the bone defect in XLH is caused not only by hypophosphatemia and altered vitamin D metabolism, but also by locally released osteoblastic mineralization inhibitory factor(s), referred to as minhibin. In our present study, we found that suppression of *PHEX* expression by *PHEX* antisense in human osteoblast cells caused an increase in cathepsin D expression at protein, but not mRNA, levels. This was associated with a decrease in cathepsin D degradation and an increased cathepsin D release into culture media. Our results also showed that lowering cathepsin D activity in antisense cell conditioned media abolished their inhibitory effect on osteoblast cell calcification, suggesting the involvement of cathepsin D in mediating the minhibin activity of the antisense cell conditioned media.

Keywords: Hypophosphatemia; Rickets; Cathepsin D; Osteoblast cell; Bone; Pepstatin

X-linked hypophosphatemia (XLH), the most common hereditary form of rickets, is an X-linked dominant disorder caused by loss-of-function mutations of *PHEX* (phosphate regulating gene with homology to endopeptidases on the *X* chromosome), leading to rachitic bone disease and hypophosphatemia with renal phosphate wasting [1]. Extensive studies in XLH patients and hypophosphatemic (*Hyp*) mice, the murine model of XLH, indicate that the bone mineralization defect in this condition is caused not only by factors extrinsic to the bone, such as hypophosphatemia and deranged vitamin D metabolism, but also by intrinsic abnormalities in bones involving the local release of osteoblastic mineralization inhibitory factor(s), referred to as minhibin(s) [2]. The exact nature of this minhibin remains illusive.

Although PHEX has been identified as a metalloproteinase and found to be expressed mostly in bone tissues including osteoblast cells (ObC), the functional role of PHEX in ObC is not known. Nevertheless, in confirming its role in the pathogenesis of XLH, we found in our previous studies that suppression of *PHEX* expression by *PHEX* antisense in MG63 cells, an established human ObC cell line, reproduced phenotypes similar to that of the primary cultured *Hyp* mouse ObC, i.e., a decrease in cellular ^{45}Ca uptake and the release of mihibin(s) into conditioned media [3].

In our present study, we found that *PHEX* antisense altered cathepsin D (Cat D) metabolism in MG63 cells with a decrease in intracellular Cat D degradation and an increase in the release of Cat D into culture media. Our results also showed that the increased Cat D

☆ *Abbreviations:* XLH, X-linked hypophosphatemia; *Hyp*, hypophosphatemic mice; Cat D, cathepsin D; ObC, osteoblast cells.

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doi:10.1016/j.bbrc.2005.04.116

activity contributed to the inhibitory effect of the antisense cell conditioned media on ObC calcification.

Materials and methods

In vitro cell cultures. The human osteoblast cell line, MG63 cells, was originally obtained from ATCC and maintained in DMEM (Gibco, Carlsbad, CA) supplemented with 7% fetal calf serum, β-glycerolphosphate (10 mM), and ascorbate (0.28 mM). The *PHEX*-sense or antisense cell lines were produced as described previously [3]. In brief, the PHEX cDNA spanning from −62 to +206 of human PHEX sequence (GenBank Accession No. U87284) was obtained by RT-PCR from MG63 cell mRNA and cloned into pcDNA 3.1 vector (Invitrogen, Carlsbad, CA) in either sense or antisense orientation. MG63 cells were stably transfected with sense or antisense vector and the G418-resistant stable transfectants were then selected and cloned to establish sense and antisense cell lines, respectively. The effect of PHEX antisense was confirmed by the suppressed expression of PHEX mRNA and protein levels in antisense cells as compared to sense cells [3].

Metabolic labeling studies. Cells were first incubated in methionine/cysteine free DMEM (Gibco) for 2 h at 37 °C and labeled overnight at 37 °C by adding Tran^{35}S-label (10 mCi/ml) (ICN Biochemicals, Irvine, CA) to DMEM. The media were then removed and cells were washed with PBS before adding DMEM containing 2 mM methionine/cysteine. After incubation at 37 °C for indicated time periods, media were collected and cells were washed and scraped in PBS, centrifuged, and the cell pellet was lysed in triple-detergent lysis buffer containing 50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1% NP-40 (wt/vol), and 0.5% sodium deoxycholate. After boiling for 10 min, immunoprecipitation of Cat D was carried out in 1.2 ml TNN solution (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, and 0.5% NP-40) containing 0.4 mg/ml pefabloc, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 5 μg/ml aprotinin, where 50 μl of protein A/G–agarose mix (Oncogene Research Product, Cambridge, MA) was added to each sample and rotated at 4 °C for 1 h. After centrifugation, the supernatants were collected and incubated with mouse anti-Cat D monoclonal antibody (Zymed Laboratories, San Francisco, CA) at 4 °C overnight, followed by adding 50 μl/ml of protein A/G–agarose mix with another incubation for 4 h at 4 °C. The antibody–protein A/G–agarose complexes were then collected by centrifugation, washed in TNN solution at 4 °C twice, resuspended in Laemmli buffer (2% SDS, 62 mM Tris, pH 6.8, 10% glycerol, and 50 mM DTT), boiled, and loaded onto SDS–PAGE. The gels were dried, exposed to X-ray films, and the Cat D-related radiobands were quantified by a densitometer (Versa-Doc, Bio-Rad, Hercules, CA). To chase Cat D degradation, cells were labeled in a similar fashion, washed with PBS, and collected after incubation in DMEM containing 2 mM methionine/cysteine and cyclohexamide (35 μg/ml) at 37 °C at 0, 2, and 4 h. Cell lysates were then obtained and processed for immunoprecipitation as described. The radioactivity of the final sample was determined by liquid scintillation spectroscopy (1600-TR; Packard, Downers Grove, IL).

Quantitative real-time PCR. Total RNA was extracted by Ultraspec RNA extract kit (Biotecx Laboratories, Houston, TX). 1.0 μg of the DNase I (Invitrogen, Carlsbad, CA) treated RNA was reverse-transcribed with random hexamer (Thermoscript RT-PCR system, Life Technologies, Rockville, MD), and PCRs were carried out with 25 ng cDNA, 150 nM each of forward and reverse primers, and 1× SyberGreen Supermix (Bio-Rad Laboratories) in a total volume of 25 μl. Samples were amplified for 40 cycles in a real-time PCR detection system (iCycler iQ, Bio-Rad Laboratories) with an initial melting at 95 °C for 8.5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR product accumulation was monitored at multiple points during each cycle by measuring the increase in fluorescence caused by the binding of SyberGreen I to double-stranded DNA. The partial cycle at which a statistically significant increase in *Cat D* product was first detected (threshold cycle, C_t) was normalized to the C_t for GAPDH. Post-amplification melting curves were performed to confirm that a single PCR product was produced in each reaction. The primer pairs used for Cat D were TTCGTCCTCCTTCGCGATT/CTCC GTCATAGTCCGACGGATA and for GAPDH were TGAACG GATTTGGCCGTATTG/ACCATGTAGTTGAGGTCAATGAAG.

Northern and Western blot analyses. Northern and Western blot analyses were performed according to standard protocols as previously described [3]. In brief, for Northern blots, cellular RNA was size fractionated on 1% agarose gels, transferred to nylon membranes, and probed with biotinyl-labeled Cat D cDNA probe. For Western blots, cell protein samples were extracted in a solution containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% Na-deoxycholate, and 1% protease inhibitor cocktail set III (Calbiochem, San Diego, CA), denatured by boiling in 2% SDS, separated on 4–15%-gradient SDS–PAGE gels, and transferred to supported nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 2% skim milk, membranes were probed with rabbit anti-mouse Cat D antisera (a generous gift from Dr. Uchiyama, Osaka University) and a secondary HRP-conjugated goat anti-rabbit IgG antibody (Pierce, Rockford, IL). The signal of the secondary antibody was visualized by chemiluminescence with SuperSingal West Femto Maximum Sensitivity substrate (Pierce) according to manufacturer's manual instruction. The same membrane, after being stripped with 9 g/dl glycine (pH 3.0), was probed for β-actin using mouse monoclonal anti-β-actin antibody (Sigma, St. Louis, MO) with a secondary alkaline-phosphatase-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotech, Santa Cruz, CA). Immunodetection was carried out with the Blue Phos Kit (Kirkegaard Perry Laboratories, Maryland).

Cat D activity assays. Conditioned media Cat D activities were determined by using a chromogenic substrate, Bz-Arg-Gly-Phe-Phe-Pro-4MeOβNA (Calbiochem, La Jolla, CA). In brief, the assay mixture (800 μl) consisted of two volumes of buffer solution (0.4 M citrate, at indicated pH) and one volume each of substrate solution and sample. The substrate was first dissolved in dimethyl sulfoxide (DMSO) and then diluted to 10 mM with water to obtain a final concentration of 1% DMSO. Incubation was carried out at 37 °C for 40 min, and the reaction was stopped with one volume of 1 mM pepstatin (Sigma). The release of 2-naphthylamine was measured in a spectrofluorometer (F-2000, Hitachi) at $\lambda_{em} = 410$ nm and $\lambda_{ex} = 335$ nm, and expressed as fluorescence unit per mg cell protein. In view of the pH-dependency of Cat D activity, these assays were conducted at pH 6.5 so as to simulate the pH value normally found in the culture medium under 5% CO_2–95% air with confluent cells.

Immunodepletion studies. To remove Cat D from conditioned media by immunodepletion, mouse anti-Cat D monoclonal antibody (Zymed Laboratories) was added to sense and antisense conditioned media at 6 μg/ml and incubated for 18 h at 4 °C followed by the addition of 90 μl/ml protein G/A–agarose and incubation for another 4 h at 4 °C. The antibody–protein G/A–agarose complexes were then removed by centrifugation, and the remaining supernatant was collected for study.

ObC ^{45}Ca incorporation assays. ObC calcification was assayed by determining ^{45}Ca incorporation within the cell layer and matrix as was previously described [3]. In brief, cells were incubated for 48 h in medium containing 0.5 μCi/ml $^{45}CaCl_2$ (ICN Biochemicals, Irvine, CA). Cell layers were then washed with Hanks' balanced salt solution and digested in 0.2 N NaOH. Aliquots of lysates were counted for ^{45}Ca activity by liquid scintillation spectroscopy (1600-TR; Packard) or analyzed for total protein using Coomassie brilliant blue G250 with BSA as the standard.

Statistical analyses. At least three determinations were obtained for each data point, and the experimental data are expressed as means ±

SEM. The significance of differences was analyzed by Student's t test for paired or unpaired data.

Results and discussion

In our present study, we found that introduction of *PHEX* antisense altered Cat D metabolism in MG63 cells with an increased Cat D protein expression. As shown in Fig. 1 (left), Western blot analyses revealed significantly higher protein levels for pro-Cat D (P, ~50 kDa) as well as the intermediate single chain-Cat D (S, ~47 kDa) and the mature heavy chain-Cat D (H, ~34 kDa) in antisense cells as compared to sense cells. This was confirmed by metabolic labeling studies where the signal intensities of these Cat D-related bands, as quantified by a densitometer, were significantly increased in cell lysates from antisense cells (Fig. 1, right). In contrast, we found that the Cat D mRNA levels, as assessed by both Northern blot analyses (Fig. 2, left) and quantitative real-time PCR (Fig. 2, right), were similar between sense and antisense cells. These results thus indicate the involvement of post-transcriptional steps in the alteration of Cat D expression in *PHEX* antisense cells. Consistent with this notion, in our pulse-chase studies where the cell lysate ^{35}S-labels were chased with unlabeled methionine and in the presence of translational inhibitor, cyclohexamide, we found a slower dissipation of the antisense cell Cat D signals down to only ~48% (i.e., ~52% degradation) after up to 4 h of chase vs. ~14% (i.e., ~86% degradation) in the sense cells (Fig. 3). It is therefore possible that the increased accumulation of Cat D proteins in *PHEX* antisense cells may at least partly be due to the decrease in Cat D degradation in these cells.

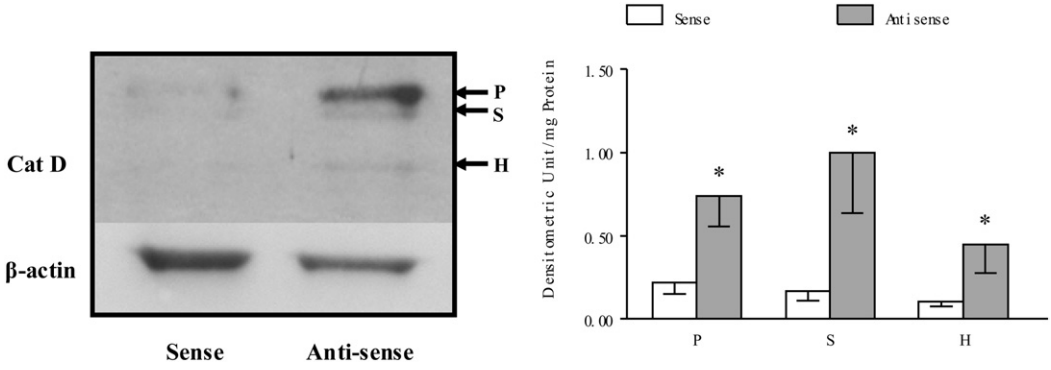

Fig. 1. Cat D protein levels in sense and antisense cells. Cat D protein levels in sense and antisense cells were evaluated by Western blot analyses (left) and metabolic labeling studies (right). For Western blots, cell protein samples were separated by SDS–PAGE and probed with rabbit anti-mouse Cat D antisera. The same membrane was stripped and probed for β-actin using anti-β-actin antibody. For metabolic labeling studies, cell lysates prepared from ^{35}S-prelabeled cells were immunoprecipitated with anti-Cat D antibody and protein A/G–agarose, followed by SDS–PAGE fluorography. The signals of radiobands were quantified by a densitometer and normalized for cell protein. Both Western blot and metabolic labeling studies showed that the protein levels for pro-Cat D (P, ~50 kDa) as well as the intermediate single chain-Cat D (S, ~47 kDa) and the mature heavy chain-Cat D (H, ~34 kDa) were significantly higher in antisense cells (shaded bars) as compared to sense cells (open bars) (means ± SE, $n = 4$, $^*p < 0.05$).

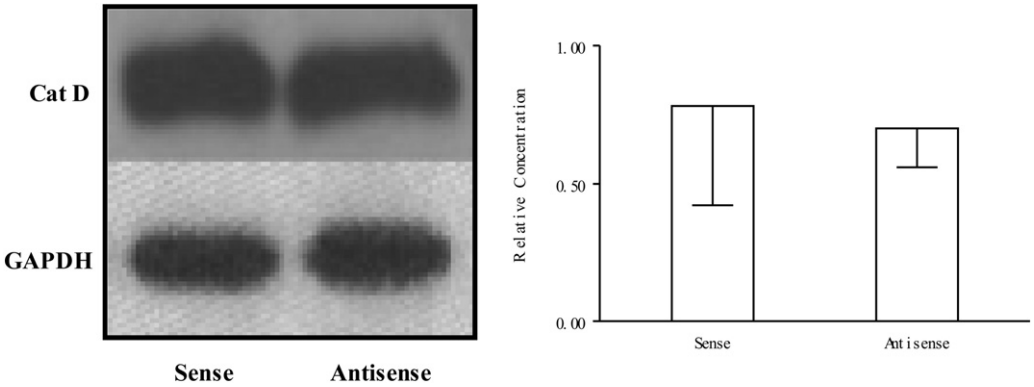

Fig. 2. Cat D mRNA levels in sense and antisense cells. The sense and antisense cell Cat D mRNA levels were evaluated by Northern blot analyses (left) and by quantitative real-time PCR (right). For Northern blots, cellular RNA samples were size fractionated and probed with biotinyl-labeled Cat D or GAPDH cDNA probe. For quantitative real-time PCR, the cDNA product from DNase-treated total RNA was used for RT-PCR and the threshold cycle (C_t) thus determined was normalized to the C_t for GAPDH and expressed as relative concentrations. Both Northern blot analyses and real-time PCR showed no difference in Cat D mRNA levels between sense and antisense samples (means ± SE, $n = 3$).

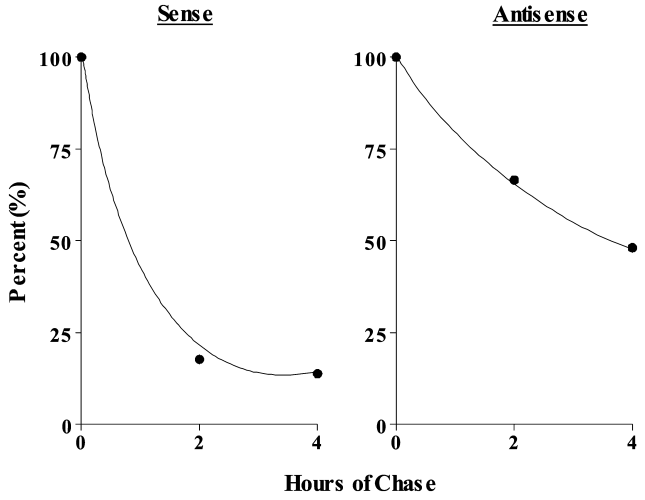

Fig. 3. Chase studies on Cat D degradation. Cells were labeled with ^{35}S overnight, washed with PBS, and collected at 0, 2, and 4 h after incubation at 37 °C in DMEM containing 2 mM methionine/cysteine and cyclohexamide (35 μg/ml). The cell lysates thus obtained were immunoprecipitated with anti-Cat D antibody and protein A/G–agarose, and the Cat D-related radioactivities from the immunoprecipitates were determined by liquid scintillation spectroscopy. As compared to sense samples, where the ^{35}S signal dissipated down to ~14% (i.e., 86% degradation) after up to 4 h chase (left), the ^{35}S signal of the antisense samples dissipated down to only ~48% (i.e., 52% degradation) (right).

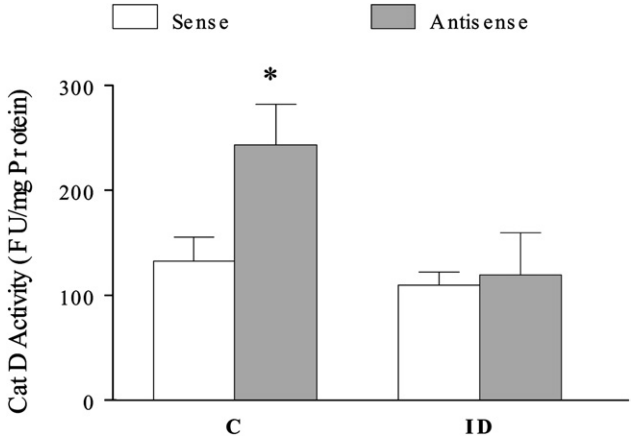

Fig. 5. Conditioned media Cat D activities. Conditioned media were collected from sense (open bars) and antisense cells (shaded bars), and the Cat D activity was determined at pH 6.5 by using a chromogenic substrate, Bz-Arg-Gly-Phe-Phe-Pro-4MeOβNA. The fluorescence of the 2-naphthylamine released was measured in a spectrofluorometer at $\lambda_{em} = 410$ nm and $\lambda_{ex} = 335$ nm, and was expressed as fluorescence unit (FU)/mg cell protein. A significantly higher Cat D activity was detected in untreated control conditioned media (C) derived from antisense cells as compared to sense cells, which was abolished when Cat D was immunodepleted by anti-Cat D antibody (ID) (means ± SE, $n = 3$, $^*p < 0.05$).

When the release of Cat D by sense and antisense cells into culture media was examined by metabolic labeling studies, we found an enhanced signal of all forms of Cat D in the antisense as compared to the sense samples (Fig. 4, left). This was demonstrated by quantifying the signal intensities of these Cat D-related bands with a densitometer and normalized for cell protein (Fig. 4, right). In correlation with this increased Cat D release by antisense cells, we were able to detect a higher Cat D activity in the conditioned media derived from these cells (Fig. 5). Since cathepsins are known to be among the major proteases involved in the degradation of bone extracellular matrix and affect bone mineralization [4], we hypothesized that the higher Cat D activity may contribute to the inhibitory effect of antisense cell conditioned media on ObC ^{45}Ca incorporation. To test this possibility, studies were performed to examine the effect of lowering Cat D activity in antisense cell conditioned media by using Cat D inhibitor, pepstatin (10 μM), or by immunodepleting Cat D with anti-Cat D antibody. Pepstatin inhibited Cat D activity to levels below detection (data not shown) and, as shown in Fig. 5, Cat D

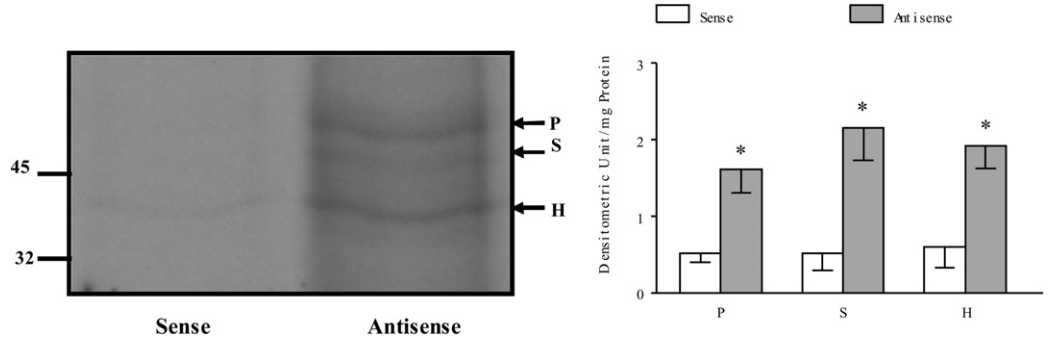

Fig. 4. Cat D release by sense and antisense cells. Cat D release by sense and antisense MG63 cells was examined by metabolic labeling studies. Cells were prelabeled with [^{35}S]methionine/cysteine and the conditioned media collected after 48 h. incubation were immunoprecipitated with Cat D antibody and protein A/G–agarose. The antibody–protein A/G–agarose complexes were then loaded onto SDS–PAGE and exposed to X-ray films. As shown by the fluorography (left), an enhanced signal of the bands corresponding to pro-Cat D (P, ~50 kDa) as well as the intermediate single chain-Cat D (S, ~47 kDa) and the mature heavy chain-Cat D (H, ~34 kDa) was detected in the antisense as compared to the sense samples. The signals of these radiobands were quantified by a densitometer and normalized for cell protein (right). The signals of all forms of Cat D were significantly higher in antisense samples (shaded bars) as compared to sense samples (open bars) (means ± SE, $n = 6$, $^*p < 0.05$).

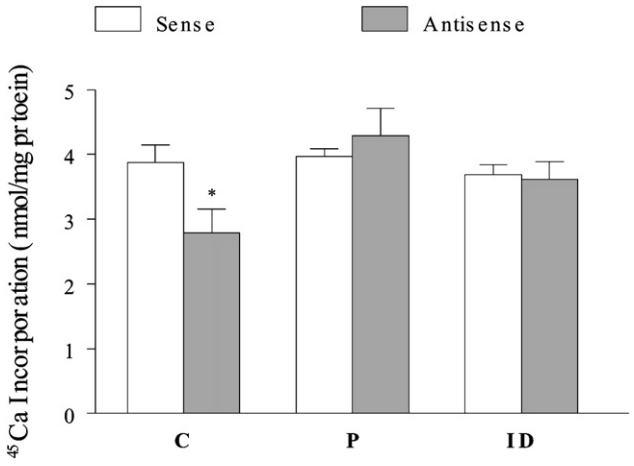

Fig. 6. Effects of pepstatin and Cat D immunodepletion in antisense conditioned media. ^{45}Ca incorporation by nontransfected MG63 cells was significantly lowered after incubation with untreated control conditioned media (C) derived from antisense cells (shaded bars) as compared to those derived from sense cells (open bars). This inhibitory effect was abolished when the higher Cat D activity in antisense conditioned media was lowered by pepstatin (10 μM) (P) or by Cat D immunodepletion (ID) (means ± SE, $n = 5$, $^{*}p < 0.05$).

immunodepletion reduced Cat D activity in antisense cell conditioned media to levels not significantly different from those of similarly treated sense cell samples. As shown in Fig. 6, while antisense cell conditioned media alone lowered MG63 cell ^{45}Ca incorporation significantly, as we previously reported [3], we found that this inhibitory effect was abolished when the higher Cat D activity in antisense cell conditioned media was lowered by pepstatin or by Cat D immunodepletion (Fig. 6). These results therefore suggest the potential role of Cat D in mediating the mineralization inhibitory effect of *PHEX* antisense cell conditioned media.

The mechanism(s) whereby Cat D inhibits ObC calcification is not immediately clear. Besides its direct action as a protease on extracellular matrix proteins, Cat D may affect a broad spectrum of cellular activities, including cell proliferation and apoptosis [5–7]. It is also to be noted that our results do not prove that the inhibition of ObC calcification is through a direct action of Cat D. In this regard, it is of interest to note that secretary proteins, such as matrix extracellular phosphoglycoprotein (MEPE) [8] and frizzled related protein 4 (FRP-4) [9], have been implicated in XLH bone defect. In particular, the inhibition of mineralization by MEPE was recently localized to a small carboxyl-terminal peptide containing the acidic serine-aspartate-rich motif (ASARM peptide) [10], and a close correlation between the serum/tissue MEPE–ASARM peptide levels and XLH phenotypes has been found [11,12]. Furthermore, by using surface plasmon resonance, Rowe et al. [13,14] have shown that PHEX binds to MEPE via this ASARM motif and protects the release of ASARM peptide from MEPE through cleavage by Cat B. Although Cat D was found to have no effect on MEPE cleavage [14], Cat D is capable of activating Cat B indirectly through its effect to inactivate cysteine protease inhibitors, cystatins [15], and directly through activation of pro-Cat B [16]. It is therefore possible that Cat D may participate in the regulation of MEPE–ASARM levels indirectly through Cat B activation. Further studies are needed to examine these possibilities.

In conclusion, we have found in our present study that, in human ObC, suppression of PHEX expression by *PHEX* antisense caused a decrease in Cat D degradation and an increase in Cat D release, which may contribute to the minhibin activity found in antisense cell condition media. The exact mechanisms whereby PHEX affects Cat D metabolism and the pathogenic role of altered Cat D metabolism in XLH bone phenotype await future studies to be clarified.

Acknowledgments

This work was supported by the grant from NIDDK (DK/AR58886). Part of this work was presented at the American Society of Nephrology meeting in 2004.

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