Increased cathepsin D release by Hyp mouse osteoblast cells

Naoko Matsumoto, Oak D. Jo, Remi N. J. Shih, Elsa J. Brochmann, Samuel S. Murray, Victor Hong, Jane Yanagawa, and Norimoto Yanagawa

Increased cathepsin D release by Hyp mouse osteoblast cells. Am J Physiol Endocrinol Metab 289: E123–E132, 2005; doi:10.1152/ajpendo.00562.2004.—The X-linked hypophosphatemia (XLH), the most common form of hereditary rickets, is 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. Available evidence today indicates that the bone defect in XLH is caused not only by hypophosphatemia and altered vitamin D metabolism but also by factor(s) locally released by osteoblast cells (ObCs). The identity of these ObC-derived pathogenic factors remains unclear. In our present study, we report our finding of a prominent protein in the culture media derived from ObC of the hypophosphatemic (Hyp) mice, a murine homolog of human XLH, which was identified as the murine procathepsin D (Cat D). By metabolic labeling studies, we further confirmed that Hyp mouse ObCs released greater amount of Cat D into culture media. This increased Cat D release by Hyp mouse ObCs was unlikely to be due to nonspecific cell damage or heterogeneous cell population and was found to be associated with an increased Cat D expression at the protein level, possibly due to a reduced Cat D degradation. However, we were not able to detect a direct effect of PHEX protein on Cat D cleavage. In support of the involvement of Cat D in mediating the inhibitory effect of Hyp mouse ObC-conditioned media on ObC mineralization, we found that exposure to Cat D inhibited ObC 45Ca incorporation and that inhibition of Cat D abolished the inhibitory effect of Hyp mouse-conditioned media on ObC calcification. In conclusion, results from our present study showed that Hyp mouse ObCs release a greater amount of Cat D, which may contribute to the inhibitory effect of Hyp mouse ObC-conditioned media on ObC mineralization.

However, despite extensive studies performed in Hyp mice and XLH patients, the etiology of the bone mineralization defect in XLH remains poorly understood. Although factors extrinsic to the bone, such as hypophosphatemia and deranged vitamin D metabolism, can contribute to the bone defect, available evidence indicates that the bone mineralization defect is also caused by intrinsic abnormalities in bones. Thus bone cross-transplant studies showed that the Hyp mouse bone defect could not be completely corrected after transplantation into the normal mouse (12–14). Similarly, ex vivo cultures of Hyp mouse osteoblast cells (ObCs) showed impaired mineralization even after prolonged cultivation (26, 42). Recent studies also showed that the restoration of Phex function to Hyp mouse ObC enabled partial correction of the bone defect despite the persistent hypophosphatemia and vitamin D abnormality (23, 3). Finally, the observation that normal mouse ObC calcification was suppressed when cocultured with Hyp mouse ObC or exposed to conditioned media derived from Hyp mouse ObC provided more direct evidence for the involvement of locally released pathogenic factor(s) (26, 42). The identity of this ObC-derived pathogenic factor(s) remains unknown.

In search of the ObC-derived pathogenic factor(s) from Hyp mouse ObC-conditioned media, we found in our present study that primary cultured Hyp mouse ObCs released greater amount of cathepsin D (Cat D) into culture media and obtained evidence implicating the role of Cat D in mediating the inhibitory effect of Hyp mouse ObC-conditioned media on ObC calcification.

METHODS

Animals. Breeding pairs of heterozygous female Hyp C57BL/6J mice and wild-type male C57BL/6J mice were originally obtained from Jackson Laboratory (Bar Harbor, ME) to maintain a continuous supply of wild-type and Hyp mice. Offspring Hyp mice were identified from the wild-type normal mice at age 4–6 wk by their characteristic Hyp phenotypes, including hypophosphatemia (2.96 ± 0.17 vs. 5.43 ± 0.21 mg/dl, n = 20; P < 0.01) and short tail length (5.88 ± 0.09 vs. 7.93 ± 0.11 cm, n = 20; P < 0.01). All mice were housed in a climate-controlled vivarium and maintained on an ad libitum diet of standard mouse chow with free access to tap water for drinking. All experiments received prior approval from our Institutional Animal Care and Use Committee.

In vitro cell cultures. The human ObC cell line, MG-63, was obtained from American Type Culture Collection (Manassas, VA), and the primary ObC cultures were grown from either femurs or calvaria obtained from normal and Hyp mice at age 6–8 wk. For ObC cultures from femurs, bones were first stripped of connective and muscle tissues aseptically and cleaned of bone marrow using steril...
PBs with a 27-gauge needle. Bone chips were then subjected to collagenase (type IV, 1 mg/ml) digestion at 24°C for 20 min and placed in 60-mm Petri dishes (Nunc Interlab, Thousand Oaks, CA). For ObC cultures from calvaria, fragments of frontal and parietal bone were aseptically removed. After suture lines and endosteme were carefully dissected away, bone fragments were positioned on top of an inverted culture plate insert (Falcon Cell Strainer; Becton-Dickinson, Franklin Lakes, NJ) in a culture dish with endocranial surfaces facing downward. ObCs were grown in DMEM (Irvine Scientific, Santa Ana, CA) supplemented with 15% fetal calf serum (Omega Scientific, Tarzana, CA), β-glycerophosphate (10 mM), and ascorbate (0.28 mM). Cells were kept in a humidified atmosphere of 5% CO2-95% air, and the culture medium was changed every 3–4 days. For serial passages, cells were trypsinized with 0.1% trypsin in Ca2+- and Mg2+-free and Mg2+-free PBS (Fisher Scientific, Pittsburgh, PA) also containing 0.5 mM EDTA and plated in appropriately sized culture plates. Cells were generally used at 2 wk after seeding and were serum deprived for 24 h before experiments. Conditioned media were collected after 48 h of incubation with confluent cells in serum-free culture media. Because both femoral and calvarial ObCs produced similar results, data from these two groups of ObCs were pooled.

**Two-dimensional SDS-PAGE and protein identification.** Two-dimensional (2D) electrophoresis was performed according to the method of O’Farrell (29). In brief, isoelectric focusing was carried out in glass tubes of inner diameter 2.0 mm using 2% pH 3.5–10 ampholines (Pharmacia, Baltimore, MD) for 9,600 V·h. To each sample, 50 ng of an IEF internal standard, tropomyosin (molecular weight 33,000, isoelectric point = 5.2) was added (arrowheads in Fig. 1). The enclosed tube gel pH gradient plot for this set of ampholines was determined with a surface pH electrode. After equilibration for 10 min in a buffer containing 10% glycerol, 50 mM dithiothreitol (DTT), 2.3% SDS, and 0.0625 M Tris (pH 6.8), the tube gel was sealed to the top of a stacking gel, which was on top of a 10% acrylamide slab gel. SDS slab gel electrophoresis was carried out for ~4 h at 12.5 mA/gel, and proteins were visualized by silver staining. The protein spots of interest were excised and digested with endoprotease Lys-C, and the amino acid sequences of the resulting peptides were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS; HHMI Protein Core, Columbia Univ., New York, NY). We analyzed the amino acid sequences thus obtained using the Swiss-Prot protein database.

**Metabolic labeling studies.** Cells were first incubated in methionine-cysteine-free DMEM (GIBCO, Carlsbad, CA) for 2 h at 37°C and labeled overnight at 37°C by adding Tran35S label (10 mCi/ml) (ICN Bioc hemicals, Irvine, CA) to DMEM. The media were removed after labeling, and cells washed with PBS before DMEM containing 2 mM methionine-cysteine was added. After incubation at 37°C for indicated time periods, media were collected, and cells were washed and scraped in PBS and centrifuged; the cell pellet was lysed in triple-detergent lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% Nonidet P-40 (wt/vol), and 0.5% sodium deoxycholate. After 10 min of boiling, Cat D immunoprecipitation was carried out in 1.2 ml of TNN solution (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40) containing 0.4 mg/ml pefabloc, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 5 μg/ml aprotinin. To each sample, 50 μl of protein A/G-agarose mix (Oncogene Research Product, Cambridge, MA) were added 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 addition of 50 μl/ml of protein A/G-agarose mix and incubation for another 4 h at 4°C. The antibody-protein A/G-agarose complexes were then collected by centrifugation and washed twice in TNN solution at 4°C and resuspended in Laemmli buffer (2% SDS, 62 mM Tris, pH 6.8, 10% glycerol, 50 mM DTT), boiled, and loaded onto SDS-PAGE. The gels were dried and exposed to X-ray films, and the Cat D-related bands were quantitated 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 for indicated time periods. Cell lysates were then obtained and proceeded for immunoprecipitation as described. The radioactivity of the final sample was determined by liquid scintillation spectroscopy (1600-TR; Packard, Downers Grove, IL).

**Calcification assays.** ObC calcification was measured by determining 45Ca incorporation within the cell layer and matrix as previously described (37). In brief, cells were incubated for 48 h in medium containing 0.5 μCi/ml of 45CaCl2 (ICN Biochemicals). Cell layers were then washed with HBSS and digested in 0.2 N NaOH. Aliquots of lysates were counted for 45Ca activity by liquid scintillation spectroscopy (1600-TR; Packard) or analyzed for total protein using Coomassie brilliant blue G250 with BSA as the standard (36). In some experiments, ObC calcification was also determined by Alizarin red-S histochemical staining. For this purpose, cell layers were fixed with ice-cold 70% ethanol for 1 h and then rinsed twice with deionized water. The cells were then stained with Alizarin red-S (40 mM, pH 4.2) for 10 min, followed by rinsing five times with deionized water.
and one wash with PBS for 15 min. After another wash with fresh PBS, the Alizarin red-S stain was extracted with 10% (wt/vol) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7) for 15 min. The extract was then transferred into a 96-well plate, and the absorbance at 562 nm was measured with a microplate reader (MRX, Dynatech Laboratories). To test the effect of conditioned media on osteoclast calcification, OOCs were incubated with conditioned media for 48 h at 37°C in a humidified atmosphere of 5% CO2-95% air before calcification analyses.

**Enzyme assays and tartrate-resistant acid phosphatase staining.** The cell alkaline phosphatase and conditioned media lactate dehydrogenase (LDH) activities were measured by a spectrophotometer (Genesys 5, Milton Roy, Rochester, NY), using the phosphatase and LDH assay kits (Sigma, St. Louis, MO), respectively, according to the manufacturer’s instructions, and the results were normalized for cell protein. To detect osteoclast cells, tartrate-resistant acid phosphatase (TRAP) staining was performed by using an acid phosphatase staining kit (Sigma), where citrate/acetone-fixed cells were incubated for 1 h at 37°C in the dark in the substrate solution containing naphthol AS-BI phosphoric acid and fast naphthol AS-BT phosphoric acid solution, with and without 1-(+)-tartrate (0.67 M, pH 5.2). Cells were then rinsed with deionized water for 3 min and stained with acid hematoxylin solution. The tartric acid-resistant phosphatase-containing osteoclast cells were not affected by tartrate and remained positively stained.

**RT-PCR and quantitative real-time PCR.** Total RNA was extracted by Ultraspec RNA extract kit (Biotex Lab, Houston, TX). For RT-PCR, cellular RNA samples were reverse transcribed with random hexamer (ThermoScript RT-PCR system; Life Technologies, Rockville, MD) and amplified in a thermocycler (GeneAmp PCR system 9700; Applied Biosystems, Foster City, CA) using primer pair specific for osteocalcin (CTCTCCTTGCACCTCTGGT/AATAGTGATACGTCGATGCAGTGGT), The amplified products were size fractionated on 2% agarose gels. The absence of genomic DNA contamination was confirmed by the lack of PCR product from non-reverse-transcribed RNA samples. For quantitative real-time PCR, 1.0 µg of DNase I (Invitrogen, Carlsbad, CA)-treated RNA was reverse-transcribed in a similar fashion, and PCR reactions were carried out with 25 ng of cDNA, 150 nM each of forward and reverse primer, and 1 µl SYBER Green Supermix (Bio-Rad) in a total volume of 25 µl. Samples were amplified for 40 cycles in a real-time PCR detection system (iCycler iQ, Bio-Rad) with an initial melt 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 SYBER Green I to double-stranded DNA. The partial cycle at which a statistically significant increase in Cat D product was first detected (threshold cycle) was normalized to the threshold cycle for GAPDH. Postamplification melting curves were performed to confirm that a single PCR product was produced in each reaction. The primer pairs used for Cat D were TCTGTCCCTCTTGAGAATGTTTGAGGGTGAGTTTGCACATGCCTAGTGG, and those for GAPDH were TGAACAGGATTTGGCGTGATTTGACCCTATGAGTGAATGAG.

**Northern and Western blot analyses.** For Northern blots, cellular RNA was size fractionated on 1% agarose gels, transferred to nylon membranes, and probed with biotinylated 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% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1% protease inhibitor cocktail set III (Calbiochem). Protein samples were denatured by boiling in 2% SDS, separated on 4–15% gradient SDS-PAGE gels, and transferred to supported nitrocellulose membranes (Millipore, Bedford, MA). The cellulose membranes were blocked with 2% skim milk and probed with rabbit anti-mouse Cat D antiserum (a generous gift from Dr. Uchiyama, Osaka University). After a washing with Tris-based saline with 0.05% Tween 20, membranes were probed with a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Pierce, Rockford, IL), and the signal of the secondary antibody was visualized by chemiluminescence with SuperSignal West Femto maximum sensitivity substrate (Pierce) according to the manufacturer’s instructions. The same membrane, after it was stripped with 9 g/dl glycine (pH 3.0), was probed for β-actin using mouse monoclonal anti-β-actin antibody (Sigma) with a secondary alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunodetection was carried out with the Blue Phos kit (Kirkegaard Perry Lab).

**Production of recombinant secreted PHEX.** A recombinant soluble, secreted form of human PHEX (secPHEX) was produced according to previously described procedures (7) with slight modifications. In brief, the human PHEX cDNA obtained from MG-63 cells, a human OBC cell line, was subjected to modifications, including the introduction of hydrophobic amino acid residues (LTVAQQ) together with the deletion of four codons (LFLV) in the transmembrane domain and the addition of c-Myc/His tags into the COOH terminus. The cDNA encoded the final secPHEX was obtained by PCR using the primer pair GGTACCGCTCTTGAGACCGACCCACAA/TCTAGACTAATGCTGCCATGGAGATGAGCTTCTCCTTCTGAGTGACGCTTCTGCTCCTATGCGCAGGATGCTCATCGT, The amplified products were ligated into pGEM-T easy vector (Promega) and the positive clones were verified by DNA sequencing and subcloned into pcDNA3.1+ expression vector (Invitrogen). To generate a catalytically inactive form of secPHEX, the pcDNA3.1+-pGEM-T/PHEX vector, harboring a Glu to Val substitution was produced by site-directed mutagenesis using the same PCR-based strategy referred to above and appropriate oligonucleotide primers. The linearized pcDNA3.1+ containing the cDNA encoding active or inactive secPHEX was then introduced into LLC-PK1 cells by electroporation (Gene Pulser II, Bio-Rad), and the stably transfected clones of LLC-PK1 cells were selected by their G418 resistance. The culture media derived from these transfected LLC-PK1 cells, were collected, and the secPHEX secreted into culture media was purified with a polyhistidine column (Talon resin; Clontech, Palo Alto, CA) according to the manufacturer’s instructions. The yield of purified secPHEX was 300 µg/l of conditioned media.

**PHEX enzymatic activity assays.** We evaluated the enzymatic activity of secPHEX thus produced by using a synthetic PHEX substrate peptide, Abz-GFSDYK(Dnp)-OH (PeptideGenic Research, Livermore, CA), as previously described (10). Abz-GFS-DYK(Dnp)-OH (10 µM) was incubated with 500 ng of secPHEX for 30 min at 37°C in 10 mM Bis-Tris buffer, pH 5.5, containing also 150 mM NaCl, and the fluorescence at emission wavelength = 420 nm and excitation wavelength = 320 nm was monitored by a spectrofluorometer (F-2000, Hitachi). The mutual effect between secPHEX and Cat D was tested by coincubation with Cat D (1 µg) with secPHEX (1 µg) under the same condition and probed with antibodies against Cat D (Zymed Laboratories) or anti-c-Myc antibody (Sigma) for secPHEX.

**Cat D activity assays.** Conditioned media Cat D activities were determined by using a chromogenic substrate, Bz-Arg-Gly-Pho-Phe-Pro-4MeOBNA (Calbiochem, La Jolla, CA), as was previously described (31). 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 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 M pepstatin (Sigma). The release of 2-naphthylamine was measured in a spectrofluorometer (F-2000, Hitachi) at emission wavelength = 410 nm and excitation wavelength = 335 nm and expressed as fluorescence unit per milligram cell protein.

**Induction of Cat D hyperexpression.** To induce Cat D hyperexpression, Cat D cDNA was first produced by RT-PCR with mRNA isolated from MG-63 cells, using primer pairs GCCGCCACCATGCAAGCCCTCCA/GGACTCTCTCTCTTCTGTCG, and ligated...
into pGEM-T easy vector, and positive clones were verified by DNA sequencing and subcloned into pcDNA3.1+ vector. The pcDNA3.1+ containing the cDNA encoding full-length Cat D was then introduced into MG-63 cells by electroporation. Control cells were transfected in a similar fashion with pcDNA3.1+ vector without the Cat D cDNA insert. The successful induction of Cat D hyperexpression was confirmed by demonstrating higher cellular Cat D mRNA abundance with Northern blot analysis (data not shown).

Immunodepletion studies. To remove Cat D from conditioned media by immunodepletion, mouse anti-Cat D monoclonal antibody (Zymed Laboratories) was added to Hyp mouse 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 A/G-agarose complexes were then removed by centrifugation, and the remaining supernatant was collected for study. As controls, separate Hyp mouse-conditioned media were treated in a similar fashion except that mouse IgG (Sigma) was used in place of mouse anti-Cat D antibody.

Statistical analyses. At least three determinations were obtained for each data point. Experimental data are expressed as means ± SE. The significance of differences was analyzed by Student’s t-test for paired or unpaired data or by one-way ANOVA with individual elements confirmed by Scheffé’s method.

RESULTS

Identification of Cat D from Hyp mouse-conditioned media. Similar to previous reports (9, 11), we found that Hyp mouse ObC cultures incorporated significantly less 45Ca within the cell layer and matrix than normal mouse ObC cultures (3.51 ± 0.25 vs. 5.07 ± 0.36 nmol/mg protein, n = 5; P < 0.05) and the Hyp mouse ObC-conditioned media was capable of inhibiting normal mouse ObC 45Ca incorporation by an average of 26.6 ± 1.1% (n = 6) compared with the normal mouse-conditioned media. Because the normal mouse-conditioned media per se were without effect on ObC 45Ca incorporation compared with the plain culture medium, we considered it likely that the inhibitory effect of the Hyp mouse-conditioned media was caused by the presence of factor(s) exerting the same bioactivity rather than the absence of factor(s) exerting the opposite bioactivity. Therefore, we searched for proteins that are either unique or prominent in Hyp mouse ObC-conditioned media compared with that of the normal mouse by comparing 2D SDS-PAGE protein patterns (Fig. 1). Among many different protein spots between normal and Hyp mouse samples, we noticed protein spots at ~50 kDa that were consistently prominent in all Hyp mouse samples (circled area in Fig. 1). These protein spots have the same molecular weight but varying isoelectric points and are likely to represent either isoforms or posttranslational modifications of a protein. These protein spots were excised, and amino acid sequences were determined by MALDI-TOF MS. We analyzed the amino acid sequences thus obtained using the Swiss-Prot protein database, and the original protein was identified as the murine precursor of Cat D, i.e., pro-Cat D (EC 3.4.235).

Metabolic labeling studies. To further confirm that Hyp mouse ObC released a greater amount of Cat D into culture media, metabolic labeling studies were performed. In these studies, the conditioned media collected from 35S-labeled normal and Hyp mouse ObCs were immunoprecipitated with anti-Cat D antibody and protein A/G-agarose, and the antibody-protein A/G-agarose complexes were analyzed by SDS-PAGE and fluorography. As shown in Fig. 2, top, an enhanced signal of the bands corresponding to pro-Cat D (~50 kDa), intermediate single-chain Cat D (~47 kDa), and mature heavy-chain Cat D (~34 kDa) was detected in the Hyp mouse-conditioned media compared with the normal mouse-conditioned media. This was further demonstrated when the signal intensities of these Cat D-related bands were quantified by a densitometer and normalized for cell protein (Fig. 2, bottom). Because both femoral and calvarial ObCs produced similar results, they were pooled together. The signals of all forms of Cat D were significantly higher in Hyp samples than in normal mouse samples (means ± SE, n = 8; *P < 0.05).
due to nonspecific cell damage or heterogeneous cell population, such as the outgrowth of cathepsin-secreting osteoclast cells.

Cat D expression levels in ObC. By Northern blot analysis, we found that the Cat D mRNA levels were not increased in Hyp mouse ObC (Fig. 5, left). This was also confirmed by quantitative real-time PCR, where the relative Cat D message level normalized against GAPDH was not significantly different between Hyp mouse and normal mouse ObC (Fig. 5, right).

In contrast, by Western immunoblot, we found that the protein levels for pro-, intermediate, and mature Cat D were significantly higher in Hyp mouse ObCs (Fig. 6, left). A similar result was also obtained with the Hyp mouse ObC lysate from metabolic labeling studies, which produced enhanced protein band signals for all three forms of Cat D (Fig. 6, right).

When the cell lysate 35S labels were chased with unlabeled methionine and in the presence of cycloheximide, we found a slower dissipation of the Hyp mouse ObC Cat D signals down to only ~40% (i.e., 60% degradation) after up to 8 h of chase vs. ~20% (i.e., 80% degradation) in the normal mouse ObC (Fig. 7). Considering the concurrent higher Cat D release from Hyp mouse ObC, these results therefore indicate that Cat D degradation is suppressed in Hyp mouse ObC and may thus contribute to the accumulation of Cat D proteins in these cells.

Lack of secPHEX effect on Cat D. From these results, the possibility may arise that the loss of Phex function in Hyp mouse ObC could lead to a decrease in Cat D degradation if Cat D were a Phex substrate. We therefore carried out studies to examine the effect of Phex protein on Cat D. For this purpose, a recombinant soluble, secPHEX, was produced as described previously (7). As shown in Fig. 8A, the secPHEX thus produced was enzymatically active against a synthetic chromogenic substrate, Abz-GFSDYK(Dnp)-OH, whereas the nonactive mutant secPHEX (G581V) was without effect. The effect of secPHEX on Cat D was then tested by coincubating secPHEX with Cat D. As shown in Fig. 8C, secPHEX (1 μg) did not cleave Cat D (1 μg) after up to 1-h incubation at 37°C. Conversely, Cat D was also without effect on secPHEX (Fig. 8B). Incubation with secPHEX (5 μg/ml) also did not affect the 35S-labeled Cat D signals, including pro-Cat D, contained in Hyp mouse-conditioned media obtained from metabolic labeling studies (Fig. 8D).

Cat D activity in conditioned media. To test whether the Cat D activity in Hyp mouse-conditioned media was increased as the result of a greater Cat D release by Hyp mouse ObCs, we measured conditioned media Cat D activity by using a chromogenic Cat D substrate, Bz-Arg-Gly-Phe-Phe-Pro-4MeO

Fig. 4. Conditioned medium lactate dehydrogenase (LDH) assays and ObC tartrate-resistant acid phosphatase (TRAP) staining. Conditioned medium LDH levels (left) were determined by using LDH assay kits and normalized for cell protein. ObC TRAP staining (right) was performed by using acid phosphatase staining kit in the presence (top) or absence (bottom) of L-(-)-tartrate (0.67 M, pH 5.2), followed by hematoxylin staining. TRAP-containing osteoclast cells are not affected by tartrate and can be identified by their positive staining (dark brown) in the presence of tartrate. There was no increase in either LDH levels (means ± SE, n = 8, P = 0.3) or TRAP-positive cells in Hyp mouse ObC cultures.
conducted at pH 6.5 so as to simulate the pH value normally found in the culture medium under 5% CO2-95% air with confluent cells. As shown in Fig. 9, a significantly higher Cat D activity was detected in Hyp mouse-conditioned media.

**Effect of Cat D on ObC calcification.** Because cathepsins are known to be the important proteases involved in bone extracellular matrix turnover (6), we tested the effect of Cat D on ObC calcification by either adding Cat D extracellularly to culture media or by raising intracellular Cat D levels through Cat D overexpression. As shown in Fig. 10, A–C, exposure to Cat D (10^{-8} M) in culture media for 48 h significantly reduced normal mouse ObC 45Ca incorporation and Alizarin red-S staining. Similarly, MG-63 cells overexpressing Cat D had significantly less 45Ca incorporation and Alizarin red-S staining than the control MG-63 cells that were similarly transfected but without Cat D cDNA insert (Fig. 10, D–F). We also found that the conditioned media collected from Cat D-overexpressing MG-63 cells were capable of inhibiting normal mouse ObC 45Ca incorporation by 19.1 ± 1.6% (n = 5) compared with the conditioned media collected from control MG-63 cells. In separate experiments, we found that ObC 32P uptake was not affected by either exposure to Cat D (10^{-8} M) or Cat D overexpression (data not shown).

**Effects of Cat D inhibition in Hyp mouse-conditioned media.** To examine the role of Cat D in mediating the inhibitory effect of Hyp mouse-conditioned media on ObC calcification, we tested the effect of inhibiting Cat D activity by either using the Cat D inhibitor pepstatin (10 μM) or immunodepleting Cat D from the conditioned media with anti-Cat D antibody. Pepstatin inhibited Cat D activity to levels below detection (data not shown), and, as shown in Fig. 9, Cat D immunodepletion reduced Cat D activity in Hyp mouse-conditioned media to a level not significantly different from normal mouse-conditioned media. We found that these two maneuvers were equally effective in averting the inhibitory effect of Hyp mouse-conditioned media on ObC 45Ca incorporation (Fig. 11).

---

**Fig. 5.** Cat D mRNA levels in normal and Hyp mouse ObC. Cat D mRNA levels in normal and Hyp mouse ObC were assessed by Northern blot analyses (left) and by quantitative real-time PCR (right). For Northern blots, cellular RNA samples were size fractionated and probed with biotinylated Cat D or GAPDH cDNA probe. For quantitative real-time PCR, the cDNA product from DNase-treated total RNA was used for PCR reactions carried out with specific primers and Syber green Supermix in a real-time PCR detection system, where the PCR product accumulation was monitored by the increase in fluorescence and the threshold cycle for Cat D product was normalized against that for GAPDH. There was no difference in Cat D mRNA levels between normal and Hyp mouse ObC by either Northern blot analyses or real-time PCR (means ± SE, n = 3; P = 0.14).

---

**Fig. 6.** Cat D protein levels in normal and Hyp mouse ObCs. Cat D protein levels in normal and Hyp mouse ObCs were assessed by Western blot analyses (left) and by metabolic labeling studies (right). For Western blots, cell protein samples were size fractionated and probed with rabbit anti-mouse Cat D antisera and a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for chemiluminescence detection. The same membrane was stripped and probed for β-actin. For metabolic labeling studies, ObC were labeled with 35S overnight, and the cell lysate samples were immunoprecipitated with anti-Cat D antibody and protein A/G-agarose, followed by SDS-PAGE fluorography. The signals of radiobands corresponding to pro-Cat D (P, 50 kDa), intermediate single-chain Cat D (S, 47 kDa), and mature heavy-chain Cat D (H, 34 kDa) were quantified by a densitometer and normalized for cell protein. In contrast to mRNA, the protein levels for all forms of Cat D were significantly higher in Hyp mouse ObCs (means ± SE, n = 5; *P < 0.05).
DISCUSSION

There is convincing evidence that the bone mineralization defect in XLH is caused not only by hypophosphatemia and deranged vitamin D metabolism but also by intrinsic bone defects involving local release of factor(s) capable of inhibiting bone mineralization. Aiming to identify these ObC-derived pathogenic factors, we compared 2D SDS-PAGE protein patterns between normal and Hyp mouse ObC-conditioned media and identified a prominent protein in Hyp mouse ObC-conditioned media as the murine pro-Cat D (circled area in Fig. 1).

This increased release of Cat D into culture medium by Hyp mouse ObC was further confirmed by metabolic labeling studies, where increased signals of Cat D-related bands, including the precursor pro-Cat D (50 kDa), the intermediate single-chain Cat D (47 kDa), and the mature heavy-chain Cat D (34 kDa), were detected in the conditioned media collected from 35S-labeled Hyp mouse ObC (Fig. 2). It is unlikely that this was due to heterogeneous cell population, because both normal and Hyp mouse ObCs exhibited similar ObC characteristics, including the time-dependent increase of alkaline phosphatase activity (Fig. 3, left) and the expression of ObC marker such as osteocalcin (Fig. 3, right). It is also unlikely that this occurred through nonspecific cell damage with leakage of this lysosomal protein because we found that the LDH level, a marker for cell damage, was not increased in Hyp mouse-conditioned media compared with normal mouse-conditioned media (Fig. 4).

Fig. 7. Chase studies on Cat D degradation. ObCs were labeled with 35S overnight, washed with PBS, and collected at 0, 2, 4, and 8 h after incubation at 37°C in DMEM containing 2 mM methionine-cysteine and cycloheximide (35 μg/ml). 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. Compared with normal samples, where the 35S signal dissipated down to ~20% (i.e., 80% degradation) after being chased for up to 8 h (left, ● with solid lines), the 35S signal of the Hyp samples dissipated down to only ~40% (i.e., 60% degradation; right). In a separate group of normal mouse samples, the dissipation rate of 35S signals was reduced when protease inhibitors (protease inhibitor cocktail set III, Calbiochem) were added during the chase period (○ with dashed line).

Fig. 8. Lack of PHEX effect on Cat D. A recombinant secreted form of human PHEX (secPHEX) was produced with its activity confirmed by using a synthetic peptide substrate Abz-GFSDYK(Dnp)-OH, where the fluorescence (AFU, artificial fluorescence units) at wavelength emission = 420 nm and wavelength excitation = 320 nm increased over a 30-min incubation at 37°C in 150 mM NaCl and 10 mM Bis-Tris buffer, pH 5.5 (A; ●). Inactive mutant PHEX (○) was without effect. The mutual effects of PHEX and Cat D were examined by coincubating secPHEX (1 μg) and Cat D (1 μg) under the same condition and probed by anti-c-Myc antibody for secPHEX (B) or anti-Cat D antibody for Cat D (C). Although both secPHEX and Cat D were effectively cleaved by trypsin (0.25%), no mutual effect between secPHEX and Cat D was found. Addition of secPHEX (5 μg/ml) to Hyp-conditioned media derived from metabolic labeling studies, which contained 35S-labeled pro-, intermediate, and mature Cat D, also did not show any effect of secPHEX (D).

Fig. 9. Conditioned media Cat D activities. Cat D activity was determined by using a chromogenic substrate, Bz-Arg-Gly-Phe-Phe-Pro-4MeOβNA. Fluorescence of the 2-naphthylamine released was measured in a spectrofluorometer at wavelength emission = 410 nm and wavelength excitation = 335 nm and expressed as fluorescence unit (FU) per mg cell protein. Open bars indicate the results of untreated samples (control, n = 7; *P < 0.01 vs. normal samples), and solid bars indicate the results of immunodepleted samples (n = 4; P = 0.13).
ditioned media (Fig. 4, left). The possibility that the increased Cat D release was due to the outgrowth of cathepsin-secreting osteoclast cells was also deemed unlikely because all mouse bones in our study were cultured under the condition favorable of ObC outgrowth, i.e., in the presence of \( /H_9252\)-glycerophosphate and ascorbate. This notion was confirmed by the lack of increased TRAP-positive cells in our \( /H_{hyp}\) mouse ObC cultures (Fig. 4, right).

We found that the increased release of Cat D by \( /H_{hyp}\) mouse ObC was associated with an increased Cat D expression at protein but not mRNA level (Figs. 5 and 6), indicating alterations at posttranscriptional steps. By chasing the cell \( /E^{35s}\)-Cat D signals after replenishing unlabeled methionine and in the presence of the translational inhibitor cyclohexamide, we found a slower dissipation of Cat D-related protein signals down to only \( /E^{40}\% (i.e., 60\% degradation)\) in \( /H_{hyp}\) mouse ObCs compared with \( /E^{20}\% (i.e., 80\% degradation)\) in normal mouse ObCs after chasing for up to 8 h (Fig. 7). These results therefore indicate a reduced Cat D degradation in \( /H_{hyp}\) mouse ObC and suggest its contribution to the accumulation of Cat D protein in \( /H_{hyp}\) mouse ObC.

Although these findings may raise the possibility that the loss of Phex function in \( /H_{hyp}\) mouse ObC could lead to a decrease in Cat D degradation if Cat D were a Phex substrate, we found no evidence for a direct action of PHEX protein on either pro- or mature Cat D (Fig. 8, B–D). The secPHEX used in our present study is similar to that reported previously (7), which contained most of its extracellular domain, including the substrate-binding catalytic site, and was confirmed to be active against the substrate Abz-GFS-DYK(Dnp)-OH (10) (Fig. 8A). Although this may not completely rule out the possibility that the recombinant secPHEX does not have the correct conformation for it to be active on Cat D, we also found no evidence for Cat D cleavage by using membrane fraction prepared from LLC-PK1 cells transfected with membrane-bound PHEX (data not shown). Because the substrates of Phex are generally considered to be small peptides that fit into the S1 pocket of PHEX protein (10), it is probably not unexpected to find that PHEX does not cleave Cat D directly. Nonetheless, despite the lack of evidence for a direct PHEX protein action on Cat D, the loss of Phex function in \( /H_{hyp}\) mouse ObC is likely to be responsible for the altered...
Cat D metabolism because we found in separate studies that suppression of PHEX expression in MG-63 cells by PHEX antisense caused similar changes in Cat D metabolism (unpublished observations). It is therefore likely that the loss of Phex function alters Cat D metabolism in Hyp mouse ObC in an indirect fashion, possibly involving other protease systems or other factors mediating the degradation of Cat D.

The reason why Hyp mouse ObC hypersecrete pro- and mature-Cat D is not immediately clear. Cat D, the lysosomal aspartic protease, is produced as a precursor, pre-pro-Cat D, which is cleaved to produce pro-Cat D and eventually targeted to lysosomes via a mannose 6-phosphate receptor (MPR)-dependent pathway (21). Unloading of the proenzyme in acidic endosomes and continuous recycling of the receptors normally ensure the proper lysosomal segregation of pro-Cat D so that only a minor portion is secreted by normal cells. However, under certain conditions, hypersecretion of pro-Cat D may occur (2). A prototypical example of cathepsin secretion under physiological condition can be found in bones where the lysosomal enzymes secreted from the ruffled border membrane of osteoclast cells are activated in the acidic microenvironment at the site of bone resorption (6). Under pathological conditions, hypersecretion of pro-Cat D has also been described in various types of tumor cells due to altered MPR-dependent or -independent pathways (9, 18). Although our present study does not identify any specific mechanism responsible for Cat D hypersecretion by Hyp mouse ObC, the fact that all forms of Cat D were equally retained indicates the involvement of a mechanism of decreased early transcript catabolism. It is possible that the accumulation of pro-Cat D protein in these cells can result in saturation of the MPR pathway and lead to increased secretion of pro-Cat D, as was found in human breast cancer cells (24). Whether Hyp mouse ObC also hypersecrete intermediate- and mature-Cat D is less clear because the apparent secretion of these forms of Cat D may occur after they were fully processed intracellularly and therefore passed through the lysosomal trafficking pathway and/or through processing of pro-Cat D extracellularly via autocatalysis (41).

Irrespective of the underlying mechanism, we were able to detect a parallel increase in Cat D activity in Hyp mouse-conditioned media (Fig. 9). It is noteworthy that, although the optimal pH for Cat D activity is generally between 3 and 5, we were able to detect a higher Cat D activity in Hyp mouse-conditioned media at pH 6.5, a pH value normally found in culture media under 5% CO2-95% air with confluent cells. It is noteworthy that conditioned media at pH 6.5, a pH value normally found in optimal pH for Cat D activity is generally between 3 and 5, we detect a parallel increase in Cat D activity in.

Whether Hyp mouse ObC also hypersecretes intermediate- and mature-Cat D is less clear because the apparent secretion of these forms of Cat D may occur after they were fully processed intracellularly and therefore passed through the lysosomal trafficking pathway and/or through processing of pro-Cat D extracellularly via autocatalysis. However, our present findings, together with others' (11), imply that the inhibitory effect of MEPE on Cat D activity may be due to the MEPE-ASARM motif (35). Because Cat D is known to be capable of inactivating cysteine protease inhibitors, cystatins (22), and activating pro-Cat B (28), Cat D may thus participate in the regulation of MEPE/ASARM levels indirectly through cathepsin B. Further studies are needed to examine these possibilities.

In conclusion, we have found in our present study that Hyp mouse ObC had increased release of Cat D and obtained results implicating the potential role of Cat D as a mineralization inhibitory factor in Hyp mouse ObC-conditioned media. Although the mechanisms responsible for Cat D hypersecretion and its inhibition on bone mineralization remain to be delineated, our present findings, together with others' (11), implicate the emergence of upregulation of a wide variety of proteases as one of the unique Hyp phenotypes. How the loss of Phex function leads to the upregulation of other protease
systems and whether these in vitro findings are applicable to the bone defects in XLH patients await future studies to be clarified.

GRANTS
This work was supported by grants (to N. Yanagawa) from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-AR58886) and the Department of Veterans Affairs.

REFERENCES