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Cloning and Complete Coding Sequence of a Novel Human Cathepsin Expressed in Giant Cells of Osteoclastomas

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ABSTRACT

A gene encoding a possible novel human cathepsin, a cysteine proteinase that is distinct from previously characterized enzymes, has been identified by differential screening of a human osteoclastoma cDNA library. This molecule, termed cathepsin X, appears to represent the human homolog of the osteoclast-expressed rabbit cathepsin OC-2. Cathepsin X (GenBank accession number U20280) is 93.9% identical to OC-2 at the amino acid level, and is 92% identical at the nucleotide level within the coding region. Cathepsin X is 52.2 and 46.9% identical to cathepsins S and L, respectively, and is therefore clearly distinct from these enzymes. Cathepsin X mRNA was localized to multinucleated giant cells within the osteoclastoma tumor by in situ hybridization. These data strongly support the hypothesis that cathepsin X represents a novel cysteine proteinase which is expressed at high levels in osteoclasts. (J Bone Miner Res 1995;10:1197–1202)

INTRODUCTION

OSTEOCLASTS ARE MULTINUCLEATED giant cells specialized for the removal of both the inorganic and organic phases of bone.[1] Dissolution of the hydroxyapatite mineral phase is dependent upon acidification of the subosteoclastic resorption lacuna[2–4] via the action of carbonic anhydrase II and a vacuolar H^+-ATPase pump.[5] The pathway(s) for degradation of the organic matrix, primarily type I collagen, are less well understood, although mounting evidence has implicated cysteine proteinases (cathepsins) as key enzymes in this process. Cathepsins B, L, N, and S degrade collagen with an activity optimum in the pH 4–5 range, an environment similar to that found in the acidified resorption lacuna.[6] Inhibitors of thiol proteases inhibit bone resorption.[7–9] Cathepsins B and L are present within osteoclasts.[10] Cathepsins cleave the telopeptide extension of collagen, thereby denaturing the molecule and permitting cathepsins as well as other enzymes with gelatinase activity to complete the degradation.[11] In this regard, 92 kD gelatinase (MMP-9) has recently been shown to be expressed in large quantities in osteoclasts.[12]

In contrast to cathepsins, the expression of neutral collagenase (MMP-1) by osteoclasts is controversial. Earlier studies failed to localize this enzyme to osteoclasts, although it was readily detected in osteoblasts stimulated by bone resorbing agents.[13] Moreover, collagenase has only minimal activity at the low pH conditions of the resorption lacunae, and inhibitors of collagenase fail to block resorption of isolated osteoclasts in vitro.[8] More recently, procollagenase was identified in rodent osteoclasts[14] and has also been reported to be associated with the osteoclastic resorption surface[15] and the underlying resorption lacunae.[16] These data suggest that collagenase may also participate in bone collagen degradation, especially in terms of osteoid removal from the surface of woven bone.

In the present studies, a differential screening approach was utilized to identify genes expressed by human osteoclasts. In the course of these studies, a novel cDNA with significant homology to cysteine proteases was identified.

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Herein we report the structural characterization of the coding region cDNA for this enzyme, which we have termed cathepsin X.

MATERIALS AND METHODS

Cells and cell culture

Human osteoclastoma tumors were obtained courtesy of Dr. Andrew Rosenberg (Department of Pathology, Massachusetts General Hospital, Boston, MA). A portion of each tumor was snap frozen in liquid nitrogen for later mRNA preparation. The remainder was dissociated by a brief trypsinization and was placed into tissue culture in medium consisting of Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 2 mM L-glutamine, and 10 mM HEPES buffer. Osteoblastic (HOS-TE85), myelomonocytic (U-937), T lymphocyte (HSB-2), neuroblastoma (SK-N-MC), pancreatic adenocarcinoma (AsPC-1), and normal skin fibroblast (CRL 1467) cell lines were purchased from ATCC (Bethesda, MD). The epithelial cell line Hep-2 was kindly provided by Dr. Margaret Duncan (Forsyth Research Institute). Whole cell RNA from human tissues was purchased from Clontech (Palo Alto, CA).

Library construction and screening

The frozen tumor was used to prepare poly(A)$^+$ mRNA (Fast-Track mRNA Isolation Kit, InVitrogen, San Diego, CA). cDNA cloning into the pcDNAII vector was carried out using a commercially available kit (Librarian, InVitrogen). Approximately 2.6×10^6 clones were obtained, >95% of which contained inserts of average length 0.6 kb. A second library was constructed in phage using the Lambda-ZAP system (InVitrogen). This library consisted of $\sim 6 \times 10^5$ clones of average insert length 1.0 kb. Libraries were titered, aliquoted, and stored frozen at −70°C.

For differential screening, clones were randomly picked from the pcDNAII library and were hand plated in triplicate on nitrocellulose filters. Mixed cDNA probes were produced from mRNA isolated from the osteoclastoma tumor and from propagated stromal cells and were labeled with $\alpha[^{32}P]$dCTP by random priming to an activity of $\sim 10^8$ CPM/μg. Filters were hybridized in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, 100 μg/ml of denatured heterologous DNA at 45°C for 20 h, and were washed at 60°C in 1× SSC. Hybridization signals were detected by autoradiography.

Clones that were reactive with the tumor probe but unreactive or only weakly reactive with the stromal cell probe were isolated. Purified DNA from these clones was rescreened in a dot blot format to confirm the original result. The reactivity of clones with mixed cDNA probes from human cell lines was then determined, including U-937, HSB-2, Hep-2, SK-N-MC, As-PC1, and CRL 1467, in a dot blot format.

DNA sequence analysis

Clones passing the above screens were sequenced by the dideoxy method[17] and DNA homology searches were carried out in GenBank/EMBL databases using DNASIS (Hitachi, Brisbane, CA). For full-length cDNA characterization, the cathepsin X probe was labeled with $\alpha[^{32}P]$dCTP used to screen the Lambda-ZAP osteoclastoma library. Positive clones were purified, and the size of inserts was determined following excision with *Eco*RI. A clone containing a full-length insert of 1.6 kb was subjected to controlled digestion with *Exo*III to generate a series of diminishing insert sizes. Sequence analysis was then carried out on both ends by the dideoxy method. Homologies with known cathepsin sequences were determined using the BLAST program at N.C.B.I.

In situ hybridization

For in situ hybridization, the 0.8 kb cathepsin X insert was subcloned into pBluescript SK, and cRNA probes were generated from the T3 (sense) and T7 (antisense) promoters, respectively. Probes were labeled with digoxygenin-UTP using the Genius System (Boehringer, Indianapolis, IN). In situ hybridization was carried out on 7 μm cryostat sections of a human osteoclastoma as described previously.[12] In brief, tissue was fixed with 4% paraformaldehyde and embedded in OCT (Miles, Inc., Kankakee, IL). The sections were rehydrated, postfixed in 4% paraformaldehyde, washed, and pretreated with 10 mM dithiothreitol, 10 mM iodoacetamide, 10 mM N-ethylmaleimide, and 0.1% thiethanolamine-HCl. Prehybridization was carried out with 50% deionized formamide, 10 mM Tris-HCl, pH 7.0, Denhardt's, 500 μg/ml of yeast tRNA, 80 μg/ml of salmon sperm DNA, 0.3M NaCl, 1 mM EDTA, and 100 mM DTT at 45°C for 2 h. Fresh hybridization solution containing 10% dextran sulfate and 1.5 ng/ml of digoxygenin-labeled cRNA probe was applied after heat denaturation. Sections were coverslipped and incubated in a moistened chamber at 45–50°C overnight. Hybridized sections were washed four times with 50% formamide and 2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) containing 10 mM DTT and 0.5% Triton X-100 at 45°C. Sections were treated with RNAse A and RNAse T1 to digest single-stranded RNA and washed four times in 2× SSC and 10 mM DTT. Hybridized probes were visualized immunologically with a digoxygenin–nucleic acid detection kit according to the manufacturer's instructions (Genius System, Boehringer Mannheim). Developed slides were photographed using a Nikon Diaphot microscope. Hybridized probes were visualized immunologically with a digoxygenin–nucleic acid detection kit according to the manufacturer's instructions. Developed slides were photographed using a Nikon Diaphot microscope.

RESULTS

Osteoclastomas consist of ~30% multinucleated tartrate-resistant acid phosphatase positive (TRAP$^+$) giant cells. These cells posess a closely similar phenotype to osteoclasts and are also capable of excavating resorption pits on bone slices.[18,19] The remainder of the tumor consists of "stromal" cells, a mixture of cell types with fibroblastic/mesen-

chymal morphology. Disaggregated tumor cells were passaged weekly for 4 weeks, at which time all multinucleated, $TRAP^+$ cells had disappeared, whereas the stromal cell elements continued to proliferate. Stromal cells were mononuclear, $TRAP^-$, and variably alkaline $phosphatase^+$.

Approximately 12×10^3 clones from the pcDNAII osteoclastoma library were replica-plated and screened by differential hybridization using mixed cDNA probes derived by reverse transcription of mRNA from either the original osteoclastoma tumor ($osteoclast^+$) or from propagated stromal cell mRNA ($osteoclast^-$). Of these clones, 195 gave a positive hybridization signal with tumor cDNA but were negative or very weakly positive with stromal cell cDNA. Most of these clones were also negative when screened with mixed cDNA probes derived from a panel of human cell lines, including myelomonocytic (U-937), T lymphocyte (HSB-2), epithelial (laryngeal carcinoma HEp-2), neuroblastoma (SK-N-MC), pancreatic adenocarcinoma (AsPC-1), and normal skin fibroblasts (CRL 1467) (data not shown).

It was expected that clones passing these screens would contain genes that were osteoclast expressed. This was confirmed by dideoxy sequence analysis, which revealed that 14/195 clones contained inserts with a sequence identical to TRAP. An even larger proportion of clones (77/195) encoded MMP-9 (92 kD type IV collagenase).[12] Both of these enzymes are highly expressed by osteoclasts, indicating the effectiveness of the differential screening procedure.

In addition, one sequence was found to possess significant homology to cathepsins from human and other species but was not identical to any known cathepsin. This sequence, tentatively termed cathepsin X, occurred with 6/195 repeats, the longest of which was 0.8 kb. Northern analysis of mRNA from the osteoclastoma tumor using a ^{32}P-labeled cathepsin X probe revealed a transcript of approximately 1.9 kb (Fig. 1). Cathepsin X mRNA was found at high levels in osteoclastoma tumor and at lower levels in kidney but was not detected in skeletal muscle, liver, or brain. Cathepsin X mRNA was also absent from osteoclastoma stromal cells as well as human cell lines U-937, HOS-TE85 (osteosarcoma), HSB-2, Hep-2, SK-N-MC, and AsPC-1. Rescreening the pcDNAII library failed to yield clones containing full-length inserts. Consequently, a second osteoclastoma library constructed in lambda-ZAP yielded 40 positive clones, two of which contained inserts >1.6 kb.

The complete nucleotide and deduced amino acid sequences of cathepsin X are presented in Fig. 2. An open reading frame of 987 bp originating with ATG was identified. This was preceded by an 18 bp portion of the 5′ untranslated region and was followed by a 592 bp 3′ untranslated region and poly(A18) for a total insert size of 1615 bp. Database searches revealed 92% homology at the nucleotide level within the coding region to a recently described cysteine proteinase termed OC-2 cloned in the rabbit.[20] Limited homology was observed with OC-2 in the 3′ untranslated region, as expected for genes from different species. Lesser degrees of homology to human cathepsins L (64%), S (63%), and B (45%) were also observed. Alignment of the deduced amino acid sequence of cathepsin X

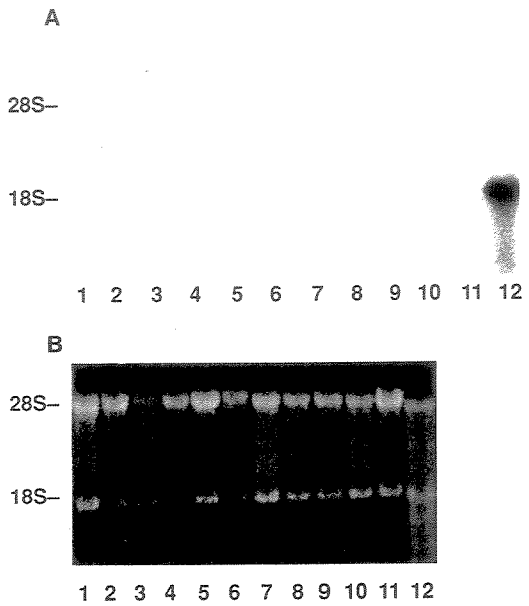

FIG. 1. Northern hybridization of cathepsin X cDNA to total cell RNA from human tissues and cell lines. Lane 1, SK-N-MC; lane 2, AsPC-1; lane 3, U-937; lane 4, HOS-TE85; lane 5, Hep-2; lane 6, HSB-2; lane 7, skeletal muscle; lane 8, liver; lane 9, kidney; lane 10, brain; lane 11, stromal cells; lane 12, osteoclastoma tumor. (A) Autoradiograph; (B) ethidium bromide stained gel.

with that of OC-2 and human cathepsins L and S is presented in Fig. 3. Again, a high degree of homology was observed with rabbit OC-2 (93.9%), with many of the differences reflecting conservative amino acid substitutions. Considerably less homology was seen with cathepsins L (46.9%) and S (52.2%). The sequence of cathespin X was submitted to GenBank (accession number U20280).

Cells within the osteoclastoma that produce mRNA for cathepsin X were identified by in situ hybridization. As shown in Fig. 4, a digoxygenin-labeled antisense probe was strongly reactive with all multinucleated osteoclasts but was unreactive with most stromal cells. In contrast, the sense probe produced only minimal background staining, which was not localized to any cell type. It was noted that a small number of mononuclear cells, possibly osteoclast precursors, also stained positively with the antisense probe. In situ hybridization with a second osteoclastoma tumor yielded an identical result (data not shown).

DISCUSSION

The precise mechanisms by which osteoclasts degrade the organic component of bone have not been determined, although it is clear that cysteine proteinases (cathepsins) play an important role in this process. In the present study, a gene encoding a possible novel human cathepsin that is distinct from previously characterized enzymes has been identified by differential screening of a human osteoclastoma cDNA library. This molecule, which we have termed

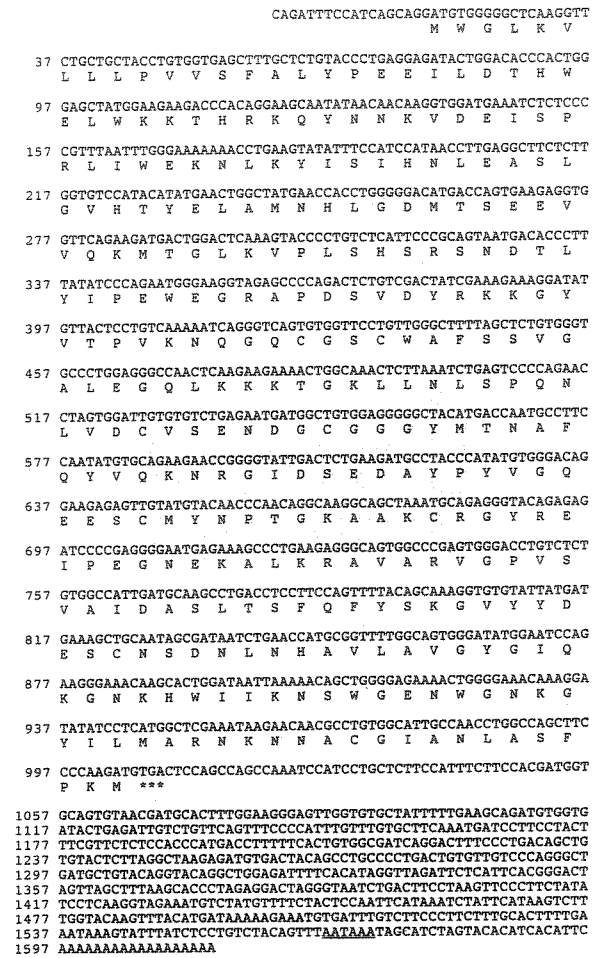

FIG. 2. Nucleotide and deduced amino acid sequences of human cathepsin X cDNA. Numbers indicate the nucleotide bases.

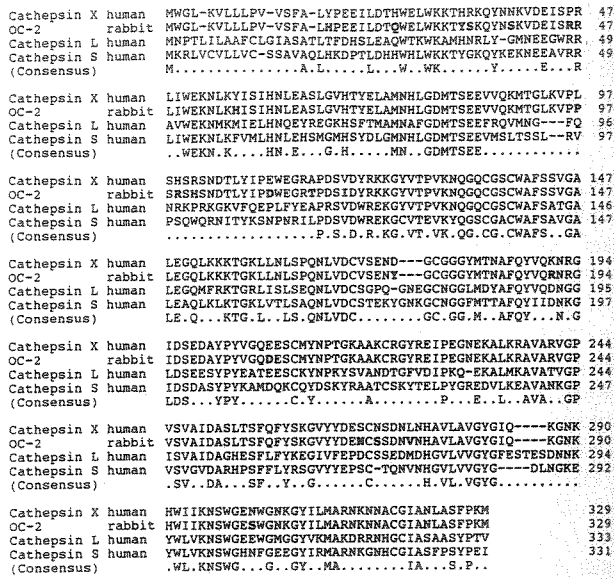

FIG. 3. Alignment of amino acid sequences of human cysteine proteinases. Gaps (dashes) introduced to maximize alignment.

cathepsin X, appears to represent the human homolog of the osteoclast-expressed rabbit cathepsin OC-2 described by Tezuka et al.[20] Cathepsin X is 93.9% similar to OC-2 at the amino acid level and 92% homologous at the nucleotide level within the coding region. As expected for molecules from different species, there is limited homology between the 3′ untranslated regions. Cathepsin X had significantly less homology to other members of the cathepsin family and is therefore clearly distinct from these enzymes. Finally, cathepsin X mRNA was localized to osteoclasts within the osteoclastoma tumor by in situ hybridization. Interestingly, cathepsin X was not detected in a number of other human cell lines, or from normal human brain or muscle, but was weakly expressed in kidney (Fig. 1). Taken together, these data strongly support the hypothesis that cathepsin X represents a novel cysteine proteinase that is distinct from previously described cathepsins and which is expressed in high levels in osteoclasts.

The previously described cathepsins B, L, N, and S possess an acidic pH optimum for the digestion of native and denatured type I collagen.[6] Of these enzymes, only cathepsins B and L have been localized immunohistochemically to osteoclasts.[22–24] Cathepsin L has generally been found to be expressed in greater amounts than cathepsin B in osteoclasts,[10,22,25] and selective inhibitors of cathepsin L, but not B, have been shown to reduce pit formation by osteoclasts.[10,25] Purification studies suggest the presence of at least two, and probably more, cathepsin-like enzymes in osteoclasts. Collagenolytic activities from homogenates of mouse calvaria have yielded cathepsins B and L, as well as a cathepsin L–like 70 kD proteinase, the latter of which may represent a complex of cathepsin L with another protein.[26] Blair et al.[27] also purified a cathepsin B–like enzyme from avian osteoclasts, which was 77% homologous to human cathepsin B. However, this enzyme represented only a minor proportion of total collagenolytic activity, suggesting the presence of additional acidic collagenases. Similarly, six separate cathepsins were purified from human osteoclastomas using biochemical approaches.[21] All six enzymes were reactive with an anti–cathepsin B antiserum, suggesting that they represent multiple forms of cathepsin B. However, these activities exhibited heterogeneity of molecular weight, enzymatic activity, and inhibition patterns. Since cathepsins share strong active site homology, it is possible that this antiserum against cathepsin B cross-reacted with other cathepsin species, including cathepsin X. Because the sequence of cathepsin N is still unknown, it has been suggested that cathepsin X might be identical to this enzyme. Weighing against that possibility is the finding that none of these six cathepsins exhibited specificity typical of cathepsin N, i.e., preferential hydrolysis of BZ-Phe-Val-Arg-NHMec vs Z-Phe-Arg-NHMec.[21]

Because we focused initially on clones producing strong signals with the mixed cDNA tumor$^+$ probe in the differential screening step, most of the genes identified are expressed at relatively high levels in osteoclasts, such as

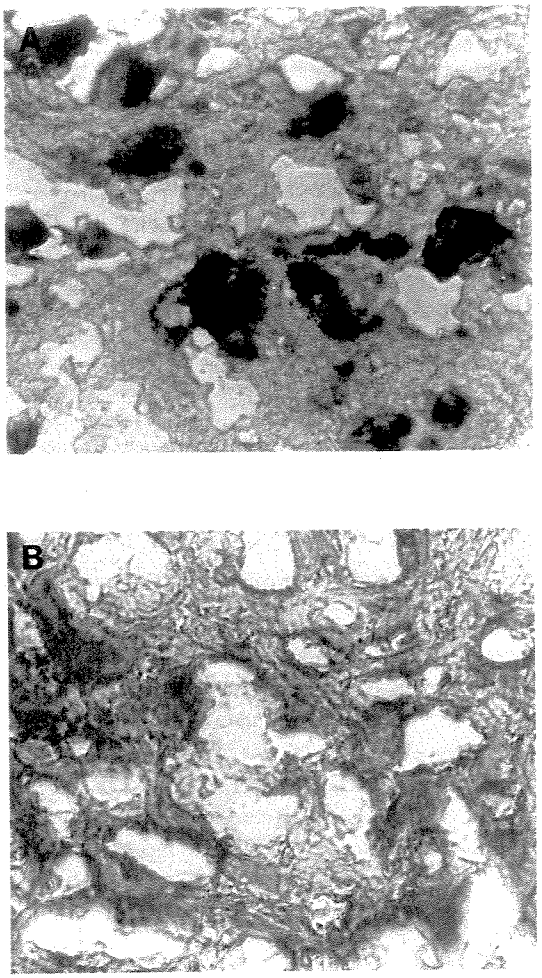

FIG. 4. In situ hybridization of cathepsin X mRNA in a human osteoclastoma. cRNA probes were digoxygenin-labeled developed with alkaline phosphatase–labeled antibody. Counterstain: methyl green. (A) antisense; (B) sense control. Magnification, ×400.

TRAP, gelatinase B, and cathepsin X. The high mRNA levels for cathepsin X in osteoclasts was further confirmed by the strong Northern blot and the in situ hybridization signals generated. Since neither cathepsin L nor B was identified by this approach, it might be speculated that cathepsin X is uniquely expressed by osteoclasts and not by other cell types within this tumor. In this regard, cathepsin B has been reported to be expressed by stromal cells as well as by osteoclasts.[28]

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NOTE ADDED IN PROOF

Since the original submission of this article, Sako and Grasser (1990 J Bone Miner Res **5**:31) have reported the application of reflected light microscopy to quantify the effects of bisphosphonates on resorption by isolated rat osteoclasts.

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NOTE ADDED IN PROOF

Since the original submission of this article, Sako and Grasser (1990 J Bone Miner Res **5**:31) have reported the application of reflected light microscopy to quantify the effects of bisphosphonates on resorption by isolated rat osteoclasts.