Molecular cloning of human cathepsin O, a novel endoproteinase and homologue of rabbit OC2

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Abstract A 1670-bp cDNA coding for a novel human cysteine protease has been isolated from a monocyte-derived macrophage cDNA library. This cDNA predicts a 329-amino acid preprocathepsin with more than 50% identity to both human cathepsin S and cathepsin L and 94% identity to a rabbit cDNA, termed OC2, recently isolated from osteoclasts. Based on its high homology to OC2, we have named the human enzyme cathepsin O. Cathepsin O mRNA was identified as a single ~1.7 kb transcript in cultures of 15-day-old monocyte-derived macrophages, but was not expressed in human monocytes or alveolar macrophages. When transfected into COS-7 cells, cathepsin O displayed potent endoproteolytic activity against fibrinogen at acid pH. This novel endoprotease may play an important role in extracellular matrix degradation.

Key words: Human cathepsin O; Endopeptidase; Amino acid sequence; Monocyte-derived macrophage; Fibrinogen; Differential hybridization

1. Introduction

Cathepsins H, L, B, and S are papain family cysteine proteinases that have been implicated in a variety of physiological processes such as proenzyme activation [1-3], enzyme inactivation [4], antigen presentation [5-7], hormone maturation [8], tissue remodeling and bone matrix resorption [9-12]. Their proteolytic activities may also be relevant to human diseases such as neoplasia [13-16], arthritis [17], emphysema [18,19], and Alzheimer's disease [20,21]. Although they share identical active site amino acids (i.e. Cys, His, and Asn [22]) as well as a high degree of homology around their catalytic domains (85-95%), these classical cysteine proteases display distinct enzymatic activities. For example, whereas cathepsins H and B are weak endopeptidases and primarily act as either an aminopeptidase or carboxypeptidase, respectively, only cathepsins S and L display strong endopeptidyl activities [23-25].

Within the papain family of cysteine proteinases, sequence data are currently available for only the four enzymes discussed above [26-30]. Prior biochemical work has suggested that additional members of this family exist [31], but little information is available regarding their structure or function. Herein, we report the isolation of a novel human cysteine protease the expression of which is dramatically up-regulated during the in vitro maturation of peripheral blood monocytes into macrophages. The human macrophage-derived cDNA bears strong homology to a putative cysteine protease recently isolated from rabbit osteoclasts [32], and the expressed protein product is shown for the first time to display potent endoproteolytic activity.

2. Materials and methods

2.1. Cell culture and RNA preparation

Human peripheral blood monocytes were adherence purified as described [33] and either harvested immediately or cultured for 15 days on 35-mm Petri dishes (Falcon) in RPMI-1640 supplemented with 40% autologous serum. Total RNA was isolated from the adherent monocytes and the 15-day-old monocyte-derived macrophages by the guanidine isothiocyanate method as described [34]. Poly(A)+ RNA was purified by oligo(dT) cellulose column chromatography [35].

2.2. Construction and differential screening of the monocyte-derived cDNA library

A cDNA library from 15-day-old monocyte-derived macrophages was prepared in AZAP Express vector (Stratagene) using the manufacturer's protocol. Approximately 1 x 10⁶ recombinant phage were differentially screened with [32P]° labeled cDNA synthesized from either adherent monocytes or monocyte-derived macrophage poly(A)+ RNA [36]. Positive plaques that preferentially hybridized to monocyte-derived macrophage-specific cDNA probes were selected and subjected to a second round of differential hybridization. Approximately 800 macrophage-specific clones remained positive after the second screening. Of the 300 clones sequenced thus far, a single clone displaying a novel sequence homology to the papain superfamily was identified for further analysis.

2.3. Cloning and sequencing of the cathepsin O cDNA

A partial length cDNA clone of cathepsin O was labeled with [α-32P]dATP using random hexamer extension (Multiprime, Amersham Corp.) and used as a probe to screen the macrophage cDNA library. Following primary, secondary and tertiary screening, individual positive plaques were isolated and resuspended in phage diluent. Insert sizes were determined by direct PCR amplification of the phage suspensions using T3 and M13 universal primers. The largest insert was automatically subcloned into pBS-CMV vector by co-infection with ExAssist helper phage according to the supplier (Stratagene). The entire insert was then sequenced by the dyeoxy chain termination method (Sequenase version 2.0, US Biochemical Corp.) as well as automatic sequencing (Applied Biosystems) for both sense and antisense strands employing a primer walking strategy. The amino acid sequence was deduced from the cDNA sequence and further aligned with rabbit OC2 and human cathepsin B, S, L or H with MacVector Version 4.1 software (Kodak, IBI).
2.4. Northern analysis

Glyoxylated total RNA (10 µg) or poly(A)⁺ RNA (1 µg) was electrophoresed on a 1.2% agarose gel and the RNA transferred to Hybond N⁺ paper (Amersham Corp.). Blots were prehybridized in 50% formamide, 5 x SSPE, 5 x Denhardt's solution, and 0.1% SDS and salmon sperm DNA at 42°C [35]. Hybridization with the [α-³²P]dATP-labeled ~800 bp cathepsin O cDNA fragment and 1.0 kb fragment of acidic ribosomal phosphoprotein [37] was carried out under identical conditions.

Fig. 1. cDNA sequence and deduced amino acid sequence of human cathepsin O. The numbers at the right side indicate the positions of nucleotides, 20 min each.

2.5. COS-7 cell transfection and endoprotease assay

The ~1.7 kb cDNA insert was released from pBK-CMV with EcoRI and XhoI (Boehringer-Mannheim) digestion and further subcloned into pcDNA-I expression vector (InVitrogen). This construct was transfected into COS-7 cell (American Type Culture Collection) with the DEAE-dextran/chloroquine method described previously [29]. Human cathepsin S was also subcloned into pcDNA-I and transfected into COS-7 cells. Two to three days post-transfection, cells were collected and lysed in buffer containing 1% of Triton X-100, 40 mM NaAc, and 1 mM EDTA, pH 4.5, for 1 h at 37°C with 10 x 10⁶ cells/ml. After centrifugation at 450 x g for 3 min, 12 µl of the supernatant was used.
for endopeptidase analysis by adding 38 μl of assay buffer containing 0.05% of Triton X-100, 20 mM NaAc, and 1 mM EDTA, pH 4.5, 1 μl of 100 mM cysteine, 50 μg/ml pepstatin A, and 125I-labeled fibrinogen at 1 mM final concentration. E-64 (10 μM) was added where indicated to inhibit cysteine protease activity. Following a 1 h incubation at 37°C, the reaction mixture was analyzed by SDS-PAGE on a 13% polyacrylamide resolving gel under reducing conditions. The electrophoresis was followed by Coomassie blue staining, destaining, gel drying, and exposure to Kodak X-OMAT film at -80°C.

3. Results

3.1. Isolation of a novel cysteine protease by differential hybridization of a human macrophage cDNA library

A cDNA library was prepared in ZAP by using total RNA isolated from 15-day-old human monocyte-derived macrophages. Duplicate plaque lifts were hybridized by using 32P-labeled first strand cDNA probes prepared from total RNA isolated from either 2-h adherent monocytes or the monocyte-derived macrophages. Of the ~300 cDNA clones preferentially expressed in the monocyte-derived macrophages that have been sequenced to date, a single ~800 bp fragment was identified that was predicted to encode a protein product with >50% identity to human cathepsin S and L as well as >95% identity to the recently sequenced rabbit OC2 isolated from rabbit osteoclasts. Furthermore, the fragment contained the triad of active site amino acids (i.e. Cys, His and Asn) characteristic of the cysteine protease family. This fragment was subsequently used to screen the macrophage cDNA library for a putative full-length insert.

3.2. cDNA library screening, DNA sequencing and amino acid sequence comparison

Approximately 50 positive clones were isolated from ~1.5 x 10^5 phages in the monocyte-derived macrophage library. The largest insert detected was ~1.7 kb and was found to contain a 1661 bp cDNA encoding a 329 amino acid protein. As shown in Fig. 1, an examination of the protein sequence identified a putative (i) 15 amino acid signal sequence, (ii) cleavage site at Arg14-Ala25 between the pro- and mature forms of the enzyme and (iii) three possible N-glycosylation sites. A ~1.7 kb cDNA O transcript was detected in monocyte-derived macrophages (but not adherent monocytes; Fig. 2) indicating that the cDNA clone is likely to be full-length. Interestingly, this transcript could not be detected in human alveolar macrophages even when the quantity of total RNA blotted was increased to 50 μg (not shown).

Amino acid sequence alignment with other known human cysteine proteinases and rabbit OC2 shows that this novel protein has 94% identity to rabbit OC2, 56% identity to human cathepsin S, 50% identity to human cathepsin L, 39% identity to cathepsin H, and only 20% identity to human cathepsin B (Fig. 3).

3.3. COS-7 cell transfection and endopeptidase assay

To determine whether human cathepsin O can express endopeptidase activity, lysates prepared from control, mock-transfected, cathepsin O-transfected or cathepsin S-transfected COS-7 cells were incubated with [125I]labeled fibrinogen at pH 4.5 for 1 h at 37°C, and degradation assessed by SDS-PAGE/fluorography. As shown in Fig. 4, the endogenous cathepsin B activity found in untransfected or mock-transfected cell lysates [30] displayed only weak proteolytic activity that could be blocked by the general cysteine protease inhibitor, E-64 (lanes 2–5). In contrast, when lysates recovered from cathepsin O-transfected cells were incubated with [125I]fibrinogen, all 3 chains of the substrate (i.e. the α, β, and γ chains), were completely degraded (lane 8). As expected, cathepsin O-mediated proteolysis was also sensitive to inhibition by E-64 (lane 9). Interestingly, despite the fact that cathepsins O and S display the highest degree of homology among the human cysteine proteinases, the pattern of degradation products generated with cathepsin S-transfected lysates was distinct from that observed with cathepsin O (compare lanes 6 and 8). Furthermore, unlike cathepsin S, cathepsin O-dependent proteolytic activity could not be detected at pH 7.0 (not shown). Together, these results indicate that cathepsin O is a new member of the human cysteine proteinase gene family.

4. Discussion

The cDNA sequence reported in this communication codes for a 329-amino acid protein with 94% identity to the rabbit OC2 cDNA sequence recently cloned from osteoclasts [32]. Because this novel protein most likely represents the human counterpart of rabbit OC2, we named it cathepsin O to reflect the relationship between the two gene products. Like cathepsin O, the reported cDNA sequence for OC2 was predicted to encode a cysteine protease. However, the rabbit enzyme was not expressed or isolated and no prior demonstration of enzymic activity had been reported. In this study, we have demonstrated that cathepsin O is a potent endopeptidase at acidic pH.
As shown in Figs. 1 and 3, human cathepsin O has much higher homology to cathepsins S and L than either cathepsin H or cathepsin B. This suggests that the enzymatic activities of cathepsin O may be more like either cathepsin S or L and less like cathepsins H and B, as is also suggested by its potent endoprotease activity. As an aside, it should be noted that it is possible that the enzyme we have termed cathepsin O bears structural resemblance or identity to a previously reported collagenolytic enzyme, cathepsin N[31]. Rabbit cathepsin N, isolated from spleen, was reported to be ~34 kDa in size. Although the predicted size of cathepsin O based on its amino acid sequence is only 24 kDa, we have detected a ~38 kDa band by SDS-PAGE analysis of rabbit cathepsin O (V.Y.R., S.M.B. and S.J.W., unpublished observation). Whether the radiolabeled species is the mature form of cathepsin O remains to be defined.

Although the cathepsin O sequence is similar to that of cathepsins L and S, its pattern of cellular expression is distinct. While human monocytes significantly increased mRNA levels for cathepsins B, L and S within a few days of in vitro culture (V.Y.R., S.M.B. and S.J.W., unpublished observation), cathepsin O mRNA levels did not increase until late in the maturation process. Similarly, human alveolar macrophages express cathepsins L and S, its pattern of cellular expression is distinct.

### Fig. 3. Amino acid alignment of human cathepsin O with other classical cathepsins and rabbit OC2.

*Fig. 3*. Amino acid alignment of human cathepsin O with other classical cathepsins and rabbit OC2. Asterisks indicate identical amino acids as in the predicted size of cathepsin O based on its amino acid sequence. Whether the radiolabeled species is the mature form of cathepsin O remains to be defined. Although the cathepsin O sequence is similar to that of cathepsins L and S, its pattern of cellular expression is distinct. While human monocytes significantly increased mRNA levels for cathepsins B, L and S within a few days of in vitro culture (V.Y.R., S.M.B. and S.J.W., unpublished observation), cathepsin O mRNA levels did not increase until late in the maturation process. Similarly, human alveolar macrophages express cathepsins B, L, and S [25,29], but cathepsin O mRNA could not be detected. Interestingly, the appearance of the cathepsin O transcript in monocyte-derived macrophages correlates with the appearance of a highly degradative phenotype in these cells (V.Y.R., S.M.B. and S.J.W., unpublished observation). The relationship between the expression of cathepsin O by cultured macrophages and their degradative potential, however, is currently unclear. Nonetheless, the demonstration that cathepsin O can express endoprotease activity suggests that the enzyme may participate in macrophage-mediated matrix remodeling and, by analogy to the pattern of expression reported for OC2,
in osteoclast-mediated bone resorption as well. Further studies are required to define those conditions wherein cathepsin O is expressed and the role that the proteinase plays in the progress of matrix-destructive conditions.

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