Human alveolar macrophages (HAM) express an elastase activity of acidic pH optimum inhibitable by cysteine protease inhibitors. Recent studies indicate that the only known eukaryotic elastinolytic cysteine protease, cathepsin L, cannot completely account for this activity. In order to search for additional cysteine proteases with elastinolytic activity, low degeneracy oligonucleotide primers based on regions of strong homology among the known cysteine proteases were used to screen reverse-transcribed HAM RNA for cysteine proteases by the polymerase chain reaction. Among the cDNA sequences generated was a 493-base pair product highly homologous to bovine cathepsin S. Screening of a HAM cDNA eukaryotic expression library with this cDNA yielded a 1.7-kilobase full-length cDNA highly homologous to bovine cathepsin S (~85% identical). This cDNA predicts a 331-amino acid preprocathepsin. Expression of this cDNA in COS cells revealed the active enzyme to be a single chain 28-kDa protease, as judged by active site labeling with a novel iodinated analogue of N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucylamido-(4-guanido)butane (E-64). The recombinant enzyme was found to be elastinolytic toward 3H-labeled elastin (bovine ligamentum nuchae) at pH 5.5 but with 25% of this activity retained at pH 7.0. Labeling of HAM with the active site probe revealed these cells express a 28-kDa cysteine protease, and Northern blot analysis revealed the presence of a ~1.7-kilobase cathepsin S mRNA. These data establish that human macrophages express at least two cysteine proteases with elastinolytic activity. The relatively broad pH range of human cathepsin S activity suggests this enzyme may contribute to the contact-dependent elastase activity of live human alveolar macrophages.

Macrophages are thought to be involved in connective tissue remodeling associated with chronic inflammation, injury, and healing (1). Part of their role in remodeling is mediated by proteolytic degradation of extracellular matrix proteins. Proteolysis of extracellular matrix elements by macrophages is a cooperative process involving proteases of serine, metallo, and cysteine classes (2). Human alveolar macrophages, for example, degrade elastin by a contact-dependent mechanism involving the neutral proteases plasminogen activator and a metalloprotease, as well as acidic enzymes of the cysteine protease class (3, 4). Plasminogen activator contributes to extracellular matrix metabolism possibly by exposing matrix elastin to contact zones with macrophages where elastolysis actually occurs and/or by activation of latent metalloproteases (5). We have previously reported, on the basis of inhibitor profiles, evidence that cysteine proteases are a major enzyme class involved in the contact-dependent elastolysis mediated by human lung macrophages (6).

To date only one eukaryotic cysteine protease, cathepsin L, has been demonstrated to be elastinolytic (7). Human alveolar macrophages express cathepsin L (8). However, recent studies indicate that cathepsin L alone cannot explain the elastinolytic activity of human alveolar macrophages. Macrophages obtained from cigarette smokers have much higher intracellular acidic elastase activity than those of nonsmokers, and yet the amounts of active cathepsin L are equivalent between nonsmoker and smoker cells (9). These data suggest that a second cathepsin, distinct from cathepsin L, could contribute to macrophage elastolysis. To identify novel cathepsins, we designed low degeneracy oligonucleotide primers based on regions of strong amino acid homology among the known cathepsins and screened reverse-transcribed human alveolar macrophage RNA for expressed cathepsins. Using this strategy we identified a previously uncharacterized human cathepsin, cathepsin S, and obtained the full cDNA by subsequent analysis of a macrophage cDNA library. Expression of this cDNA in COS cells revealed an enzyme of 28 kDa with elastase activity, confirming the expression of a second acidic elastase by these cells.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Reagents were obtained from Sigma with the following exceptions. Tris, glycine, sodium dodecyl sulfate, and acrylamide were electrophoresis grade from Bio-Rad. Carbobenzoxy-tyrosine-alanine-diazomethane (Cbz'-Tyr-Ala-CHNp) and carbo- benzoxycarbonyl-phenylalanine-phenylalanine-diazomethane (Cbz'-Phe-Ala-CHNp) were gifts of Dr. Robert Mason (Virginia Tech, Blacksburg, VA). Carboxbenzoxycarbonyl-phenylalanine-phenylalanine-diazomethane (Cbz'-Phe-Ala-CHNp) was obtained from Enzyme Systems Products (Livermore, CA). Cystatin C was a kind gift of Dr. Anders Grub (Lund, Sweden).

**Polymerase Chain Reaction Screening**—Two degenerate oligonucleotides were designed based on highly conserved amino acid sequences among the known cysteine proteases (underlined in Fig. 1).

**Primer 1:** 5'-TGT GCC TCT TGC TGG GTT CT-3'
**Sense**

**Primer 2:** 5'-CT GTT TTT CAC AAG CCA GTA-3'
**Anti-sense**

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§ To whom correspondence should be addressed: Respiratory Division, Brigham and Women’s Hospital, 75 Francis St., Boston, MA 02115. Tel.: 617-732-6074; Fax: 617-732-7421.

The abbreviations used are: Cbz, carboxbenzoxycarbonyl; DTT, dithiothreitol; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); Boc, tert-butoxycarbonyl; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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**Molecular Cloning and Expression of Human Alveolar Macrophage Cathepsin S, an Elastinolytic Cysteine Protease**

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Primer 1 was based on the conserved active site region; primer 2 was designed from another conserved region near the 3' end of all known eukaryotic cysteine proteases (10-14). In order to minimize the degeneracy of the pool of oligonucleotides coding for the selected amino acid sequences (>1000-fold for each sequence), the codon usage of known human and bovine cathepsin cDNAs for these sequences was employed (10-14). Human alveolar macrophage total RNA was prepared as previously described (15). One µg of RNA was heated to 65°C for 3 min and then reverse transcribed in 50 µl of reaction solution: 5 µl of 5 X RT buffer (GIBCO), 2.5 µl of 10 mM dNTPs (Pharmacia LKB Biotechnology Inc.), 3 µl of antisense primer 2 (0.4 µg/µl), 1 µl of RNasin (GIBCO), 1.5 µl of bovine serum albumin (GIBCO), and 600 units of Moloney murine leukemia virus (GIBCO). This mixture was kept at room temperature for 10 min, 42°C for 1 h, and 95°C for 5 min. PCRs were done using 5 µl of reverse transcription mixture and 5 µl of primer 1 (0.5 µg/µl), 3 µl of primer 2 (0.4 µg/µl), 2 µl of 10 mM dNTPs, 5 µl of dimethyl sulfoxide (Sigma), 10 µl of 10 X PCR buffer (Promega), 2.5 units of Taq DNA polymerase (Promega), and H2O to 100 µl. The PCR temperature cycle was 94°C x 1 min, 55°C x 2 min, 72°C x 2.5 min, repeated for 30 cycles. Fifty µl of the PCR reaction was transferred on a Promega Hybond membrane, and colony hybridization screening according to standard methods (16). Ten positive clones were selected from the secondary screening and preliminary DNA sequencing performed. Three of these clones had 453-bp insert sequences that are 86% identical to bovine cathepin S (10).

**Human Alveolar Macrophage cDNA Library Screening**—A human macrophage eukaryotic expression library (pcDNA I) was commercially prepared (Invitrogen) as previously described (15). The 493-bp PCR product was labeled with [α-32P]dATP by random hexamer extension (Multiprime, Amershan Corp.) and used for colony hybridization screening according to standard methods (16). Ten positive candidates were selected from the secondary screening and preliminary DNA sequencing performed. Three of these candidate clones were found to have sequences that exactly matched the 493-bp PCR product internal to the degenerate primers. Only one was in the correct orientation for expression. The coding region of this clone was subsequently fully sequenced in both strands using overlapping primers. When digested with pCRII TA polynkrier restriction enzymes, BamHI and XbaI (Boehringer Mannheim), this clone had a -1.7-kb insert.

**Protein Expression in COS Cells**—COS-7 cells (ATCC) were subcultured overnight in 90-mm plastic tissue culture dishes in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine serum (GIBCO). Transfection was done by a DEAE dextran/ chloroquine method (17). Preliminary experiments indicated that 30 µg of plasmid DNA was used to transfect COS-7 cells (ATCC). The recovered DNA was subcloned using the pCRlOOO TA cloning system (Invitrogen). Miniprep DNA was prepared from 55 white colonies and digested with pCRlOOO polylinker restriction enzymes. Two of these clones have 493-bp insert sequences and are 86% identical to bovine cathepin S (10).

**Human Macrophage Cathepsin S cDNA**—Based on two highly conserved regions within previously sequenced cysteine proteases, low degeneracy primers were designed and used to amplify reverse-transcribed macrophage RNA by PCR. Electrophoresis of the PCR products revealed multiple bands of ~500 bp. After subcloning these DNAs as a mixture into pCRII1000 and subsequent DNA sequencing of clones containing inserts, cDNA sequences were obtained that are 86% identical to those of the known human macrophage cysteine proteases, i.e. cathepsins B, H, and L (data not shown). In addition a 493-bp cDNA product showing 86% identity to the...
corresponding region of bovine spleen cathepsin S was observed (10). This cDNA was used to screen a human alveolar macrophage cDNA library. The entire coding region of one full-length clone has been extensively sequenced using vector and internal primers (Fig. 1). This cDNA predicts a 331-amino acid preprocathepsin. Compared with available partial bovine cathepsin S sequences, it shows 85 and 87% identity at the amino acid and cDNA levels, respectively (10).

Northern Blot Analysis of Human Alveolar Macrophage Cathepsin S mRNA—Fig. 2 illustrates the reaction of the 32P-labeled cathepsin S cDNA (493-bp PCR product) with human alveolar macrophage RNA. Cathepsin S mRNA was detected as a single band migrating at ~1.7 kb, consistent with the size of human cathepsin S predicted by cDNA cloning and that reported for bovine cathepsin S mRNA (10). The specificity of the cathepsin S cDNA hybridization was verified by reprobing the filter with a 32P-labeled cathepsin L cDNA (not shown). This revealed a single, distinct band slightly lower than cathepsin S, consistent with the reported size of cathepsin L mRNA, ~1.5 kb (12), and indicated that the cathepsin S cDNA was not detecting other cathepsin mRNAs under the hybridization conditions employed. Among the known cathepsins, cathepsin L has the closest sequence homology both within the region probed (57% identical) and overall (49%) to cathepsin S.

**COS Cell Expression of Human Macrophage Cathepsin S**—Minirep DNA (3 μg) from clones containing 1.7-kb inserts was transfected into subconfluent COS cells. Expression of enzyme was assessed by active site labeling of COS cell lysates (pH 5.5) with 125I-labeled JPM 565. Labeled lysates were then analyzed by SDS gel electrophoresis and autoradiography of dried gels. As illustrated in Fig. 3, untransfected COS cells or COS cells undergoing sham transfection with DNA clones contain-
sequenced and found to be the single chain form of cathepsin S (data not shown). The lower molecular weight band seen in lane 1, and to a lesser extent in lanes 2–4, is also consistent with cathepsin S in its two-chain form in which the ~6-kDa light chain contains the active site cysteine (13). In contrast, COS cells transfected with macrophage cathepsin S cDNA in the correct orientation (lane 5) express a prominent labeled band at 28 kDa. Interestingly, in these cells the expression of the 33-kDa protein as an active enzyme is largely blocked.

Lysate from each transfectant was also assayed in a 3H-labeled elastin plate assay, and these data (expressed as percentages of residual activity for each inhibitor compared with lysate only controls) are shown in Table I. All tested inhibitors of cysteine proteases blocked enzyme activity to some degree. Interestingly, the relative potency of the various diazomethylketone inhibitors is the same as that previously reported for these inhibitors against purified human cathepsin L (21). Also of note, the relative potency of these inhibitors on the acidic elastase activity of alveolar macrophage lysates is shown for comparison. All tested inhibitors of cysteine proteases blocked enzyme activity to some degree.

**DISCUSSION**

Molecular cloning of human cathepsin S has established its expression by human alveolar macrophages and defined functional features of the enzyme not previously appreciated. Macrophage cathepsin S cDNA predicts a 331-amino acid preprocathepsin. Expression of this cDNA (Fig. 3) indicates the active enzyme is 28 kDa. This contrasts with the reported size of 23 kDa for active bovine cathepsin S (22). Based on these size considerations, inspection of Fig. 1 indicates active human cathepsin S has an additional 35–40 amino acids amino-terminal to the start residue of bovine cathepsin S. Of note, this amino-terminal sequence contains the only acceptor site for N-linked glycosylation (residue 104) in the entire coding region. Such sites are thought to be necessary for post-translation mannosylation important to channeling of lysosomal enzymes into lysosomes (23). Since the reported sequences for active bovine cathepsin S do not show an N-linked glycosylation site, these data indicate an additional 19 amino acids are present in human cathepsin S that are absent in bovine cathepsin S. Of note, this additional sequence is also absent in recombinant human cathepsin S expressed in COS cells (24). Previous in vitro studies have shown that this additional sequence is required for efficient folding of the enzyme (25, 26). Therefore, the lack of this sequence in recombinant human cathepsin S expressed in COS cells may explain why this enzyme is not efficiently folded in vitro (24).

**Table I**

| Inhibitor                  | Concentration | Percent inhibition |
|----------------------------|---------------|--------------------|
| None                       | 0             | 0                  |
| Cbz-Phe-Phe-CHN$_2$        | 1             | 98                 |
| Cbz-Tyr-Ala-CHN$_2$        | 1             | 88                 |
| Cbz-Tyr-Tyr(o-butyl)-CHN$_2$| 1             | 78                 |
| E-64                       | 1             | 96                 |
| Cystatin C                | 1             | 100                |

The effect of pH and protease inhibitors on recombinant cathepsin S elastase activity—Data in Table I show the effect of various known inhibitors of cysteine proteases on the elastinolytic activity of human cathepsin S expressed in COS cells. The effect of these inhibitors on the elastinolytic activity of alveolar macrophage lysates is shown for comparison. All tested inhibitors of cysteine proteases blocked enzyme activity to some degree. Interestingly, the relative potency of the various diazomethylketone inhibitors is the same as that previously reported for these inhibitors against purified human cathepsin L (21). Also of note, the relative potency of these inhibitors on the acidic elastase activity of alveolar macrophage lysates is the same as for cathepsin S, although the percentage of inhibition is lower for the macrophage lysates than the COS cell transfectants.

Previously reported data indicate that bovine cathepsin S has activity over a relatively broad range of pH (22). To determine the pH dependence of the elastinolytic activity of recombinant human cathepsin S, aliquots of COS cell transfected lysates were mixed with various pH buffers, and residual elastase activity was assessed in a 24-h assay. Although activity decreased with increasing pH of the buffers, elastinolytic activity ~25% of maximum was still measurable at pH 7 and was completely inhibited by 1 µM E-64.

**Fig. 2.** Northern blot analysis of human alveolar macrophage RNA. Human alveolar macrophage total RNA (20 µg) was separated on an agarose/formaldehyde gel, blotted to a nylon membrane, and probed using the 32P-labeled cathepsin S PCR fragment. Cathepsin S mRNA is detected as a band of ~1.7 kilobases. Positions of the 18 and 28 S ribosomal RNAs used to estimate the mRNA size were determined by UV shadowing.

**Fig. 3.** Coexpression of cathepsin S and elastase activity in COS cells. COS cells were transfected with cathepsin S plasmid, or controls, as described under "Experimental Procedures." After 3 days of culture post-transfection, cells were lysed in 1% Triton X-100, 40 mM sodium acetate, 1 mM EDTA, pH 5.5, labeled with the cysteine protease active site probe 125I-labeled JPM-565, and subjected to SDS-PAGE under reducing conditions and autoradiography. Human alveolar macrophages (obtained from a cigarette smoker) were lysed and labeled in a similar manner for comparison. Additional COS cell lysate was assayed for elastase activity against insoluble, tritiated elastin as described under "Experimental Procedures." After 3 days of culture post-transfection, cells were lysed in 1% Triton X-100, 40 mM sodium acetate, 1 mM EDTA, pH 5.5, labeled with the cysteine protease active site probe 125I-labeled JPM-565, and subjected to SDS-PAGE under reducing conditions and autoradiography. Human alveolar macrophages (obtained from a cigarette smoker) were lysed and labeled in a similar manner for comparison.

**Table 3.** Inhibitor profile of recombinant human cathepsin S expressed in COS cells

Human alveolar macrophages or COS cell transfectants expressing human cathepsin S were lysed at pH 5.5 in 40 mM sodium acetate containing 1% Triton X-100. Aliquots of lysates were mixed with various inhibitors at the indicated final concentrations and incubated with 3H-labeled elastin for 24 h. Amounts of macrophage lysate used were chosen such that the total elastin degraded by both COS cell and macrophage lysates was comparable. Data are expressed as the percentage of residual activity for each inhibitor compared with lysate only controls.
glycosyl acceptor site, it seems likely that incompletely processed forms of bovine cathepsin S with sequence homology to the human enzyme exist and promote lysosomal channeling. Currently no sequence information for precursor forms of bovine cathepsin S are available to test this prediction.

Demonstration of the elastinolytic activity of human cathepsin S suggests this enzyme is closely related to cathepsin L, the other known cathepsin with elastinolytic activity. Indeed human cathepsin S shows closest sequence homology to cathepsin L (49%) and less homology to cathepsins H (31%) and B (23%). This functional and structural similarity is also reflected by the inhibitor profile of recombinant cathepsin S (Table 1). Cbz-Phe-Phe-CHN₂ is a good inhibitor of cathepsin L but only weakly inhibits cathepsin B (24). Cbz-Phe-Phe-CHN₂ was observed to be an effective inhibitor of cathepsin S. Of note, in prior studies of elastin degradation by human cathepsin L, it was observed that no detectable elastin degradation occurred above a pH of 6.0 (8). In contrast, using similar buffer conditions to that of the prior work, we found cathepsin S to retain 25% of maximal elastinolytic activity at pH 7–8.

We have previously reported that lysates (pH 5.5) of human alveolar macrophages have elastase activity (9), and data shown in Fig. 3 confirm that these lysates contain a 28-kDa cysteine protease. Moreover the lysate elastase activity and the 28-kDa enzyme co-purify, suggesting this 28-kDa enzyme is cathepsin S. However, data in Table I show similarities and differences between the inhibitor profile of recombinant cathepsin S and macrophage lysate elastase activities. Activities in both the COS cell lysates containing cathepsin S and in the macrophage lysates are completely inhibited by the class-specific inhibitor, E-64, and by cystatin C. In contrast, only 20% or less of the macrophage lysate activity was inhibitable by either Cbz-Tyr-Ala-CHN₂ or Cbz-Tyr-Tyr(o-butyl)-CHN₂, whereas over 75% of the cathepsin S activity was blocked by either of these inhibitors. We suspect that these differences reflect larger amounts of active cathepsin B in macrophage lysates (Fig. 3) as compared with the COS cell lysates (Fig. 3, lane 5) competing for binding with these inhibitors. It is also possible that there are additional cysteine proteases in the macrophage lysates. However, cathepsin S appears to be the major elastase active in these macrophage lysates.

Demonstration of the elastinolytic activity of cathepsin S establishes that human alveolar macrophages express at least three elastinolytic proteases: a 92-kDa gelatinase (25), cathepsin L, and cathepsin S. These cells thus have the potential to mediate focal elastin degradation in tissues, and such degradation has been documented in vivo (26, 27). Future studies are needed to explore the extent to which cigarette smoking amplifies cathepsin S activity in the microenvironment of lung macrophages over that of nonsmoker cells and to define the relative contribution of cathepsin S to the overall elastinolytic activity of these cells. The finding that human cathepsin S has appreciable elastinolytic activity at both acidic and neutral pH suggests that, at least among the cathepsins, this enzyme may be uniquely tailored to function at surface contact sites of cells and elastin where the interfacial pH could be expected to vary between acidic and neutral.

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