Manipulating substrate and pH in zymography protocols selectively distinguishes cathepsins K, L, S, and V activity in cells and tissues

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ARTICLE INFO

ABSTRACT

Cathepsins K, L, S, and V are cysteine proteases that have been implicated in tissue-destructive diseases such as atherosclerosis, tumor metastasis, and osteoporosis. Among these four cathepsins are the most powerful human collagenases and elastases, and they share 60% sequence homology. Proper quantification of mature, active cathepsins has been confounded by inhibitor and reporter substrate cross-reactivity, but is necessary to develop properly dosed therapeutic applications. Here, we detail a method of multiplex cathepsin zymography to detect and distinguish the activity of mature cathepsins K, L, S, and V by exploiting differences in individual cathepsin substrate preferences, pH effects, and electrophoretic mobility under non-reducing conditions. Specific identification of cathepsins K, L, S, and V in one cell/tissue extract was obtained with cathepsin K (37 kDa), V (35 kDa), S (25 kDa), and L (20 kDa) under non-reducing conditions. Cathepsin K activity disappeared and V remained when incubated at pH 4 instead of 6. Application of this antibody free, species independent, and medium-throughput method was demonstrated with primary human monocyte-derived macrophages and osteoclasts, endothelial cells stimulated with inflammatory cytokines, and normal and cancer lung tissues, which identified elevated cathepsin V in lung cancer.

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Introduction

Cathepsins K, L, S, and V are members of the lysosomal cysteine cathepsin family that have been implicated in a number of pathological roles. Among the four, they share 60% sequence homology [1–3] but each has unique properties and different homeostatic functions. Cathepsin S is a potent elastase notable for its activity at neutral pH [1]. Human macrophages use cathepsin S for MHC and antigen processing, but in disease, it is involved in atherosclerosis [1,4–6], emphysema [1], abdominal aortic aneurysms [1,7,8], arthritis [9], and other diseases associated with elastinolytic remodeling [10]. Cathepsin K serves a critical role in bone resorption and is the most potent mammalian collagenase described [11]. It was shown to solubilize collagen from adult cortical bone almost to a similar level as bacterial collagenase, and far beyond that of MMP[1]-9, -1, and -13. It has the additional proteolytic capability of cleaving type I collagen both intrahelically and at the telopeptides where other mammalian collagenases can only cleave at one site or the other [11]. Cathepsin K is also involved in cardiovascular disease, osteoporosis, and arthritis [5,12–14] and upregulated in breast and prostate cancer [15,16]. Like cathepsin S, cathepsin K also has strong elastase activity, but its activity diminishes at neutral pH [1]. Cathepsin V was first identified in the human thymus, testis, and macrophages and has been identified recently as the most potent mammalian elastase with elastolytic activity higher than cathepsins K and S, and pancreatic and leukocyte elastases by 2–8 fold [17]. Cathepsin V also is expressed in colon and breast carcinomas [18]. It is important to note that human cathepsin V has an 80% homologous sequence with human cathepsin L, and it has been shown that human cathepsin V, not human cathepsin L, is orthologous to mouse cathepsin L [2,3].

Most of these cathepsins have been defined with cell- or tissue-specific expression under normal physiology [1,3,19–22], but in disease states are turned on by a number of other cell types. In doing so, post-translational processing is altered. Glycosylation that normally occurs to target cathepsins for sorting to lysosomes [23], propeptide cleavage to activate the enzymes [24,25], and other changes affect enzyme structure and ultimately their electrophoretic migration distance in a non-reduced preparation which relies solely on SDS to add negative charge and partially denature the protein. Additionally, cathepsins have disulfide bonds that may differ between family members [12,26].

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[1] *Abbreviations used:* SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MMP, matrix metalloproteinase; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay.

Proper measurement of changes in levels of cathepsin activity in disease states would be beneficial to understand the roles of cathepsins and to develop therapies. The sequence homology of cathepsins K, L, S, and V, instability of the mature form at neutral pH, and substrate promiscuity all confuse detection and distinction of individual cathepsin activities by current and traditional methods. Fluorogenic substrates and active site labeling probes have been used to advantage and improved sensitivity [27–30], but structural similarities between family members still impede desired specificity when used with cells or mixtures of different cathepsin family members.

Here we detail a method of zymography, or substrate gel electrophoresis, to detect the activity of cathepsins K, L, S, and V from one cell extract/preparation. Zymography is a method whereby a substrate is polymerized into a polyacrylamide gel such that, upon activation, the enzymes hydrolyze the embedded substrate in situ, and proteolytic activity can be visualized as cleared bands on a Coomassie stained background. This technique has many benefits: (1) it does not require antibodies making it relatively inexpensive and species-independent, (2) separation of proteins by molecular mass and non-reducing electrophoretic migration visually confirm enzyme identity, (3) densitometry can be used for quantitative analysis, and (4) pH change can confirm specific cathepsin activity. Cathepsin L zymography protocols have been described [31–33], and in a recent study, we reported that cathepsin K activity is detectable at femtomole quantities through gelatin zymography [34]. This study is the first report of a zymography protocol for cathepsin S and cathepsin V, and we further describe slight modifications that allow for specific determination and quantification of cathepsins K, S, L, and V in cell and tissue extracts. Then we apply this method to monocyte derived macrophages and osteoclasts, endothelial cells exposed to the inflammatory cytokine TNFα, and normal and cancerous human lung tissue to demonstrate its utility in detecting cathepsins in health and disease states.

Materials and methods

Materials

Recombinant human cathepsin K isolated from insect cells (Enzo); recombinant human cathepsin K from *E. coli* (EMD Bioscience); human cathepsin L isolated from human liver (Enzo); recombinant human cathepsin S from *E. coli* (EMD Biosciences); recombinant human cathepsin S from insect cells (Enzo); recombinant human cathepsin V from NSO cells (Enzo); Cathepsin V with mutated glycosylation site was expressed in *P. pastoris* and was a kind gift from Dieter Brömme; E64 protease inhibitor (EMD Biosciences); Murine macrophage RAW 264.7 cell line (ATCC); Human breast and lung tissue lysates (Protein Biotechnologies). Tumor necrosis factor alpha (TNFα; Invitrogen), Macrophage colony stimulating factor (M-CSF; Peprotech), and receptor activator of nuclear factor kappa B ligand (RANKL).

Cell culture

Murine macrophage RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Medium (Lonza) containing 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. Human aortic endothelial cells (ECs) (Lonza) were cultured in MCDB medium 131 (Mediatech) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 1% endothelial cell growth serum (ECGS). ECs were stimulated with or without 10 ng/mL TNFα (Invitrogen) for 20 h. Cells were maintained with 5% CO_2 at 37 °C.

Primary monocyte isolation

This study was approved by an institutional review board committee and the subjects gave informed consent. Whole blood samples obtained from consenting donors were centrifuged against a Ficoll-Paque density gradient (density: 1.077 g/mL; GE Healthcare) for 30 min at 900 g to separate the buffy coat layer. After centrifugation, peripheral blood mononuclear cells (PBMCs) were aspirated, washed in PBS, and pelleted by centrifugation for 10 min. The isolated cells were then washed with a red blood cell (RBC) lysis buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, and 0.0037% EDTA) for seven minutes to remove any contaminating RBCs. The PBMCs were then washed in sterile PBS, and cell number and viability were determined using a Vi-Cell (Beckman Coulter). Monocytes were isolated by adhesion, and then differentiated into either macrophages with 30 ng/μl M-CSF in RPMI or osteoclasts using 30 ng/μl M-CSF and 30 ng/μl RANKL in alpha-MEM for 14 days. Lysates were collected and equal amounts of protein were loaded for cathepsin zymography.

Cathepsin zymography

This protocol is based on our previously published protocol [34]. All recombinant cathepsins are from human sequences. Procathepsins K and V from NSO cells (Enzo) were activated using 100 mM sodium acetate buffer, pH 3.9, 10 mM DTT, and 5 mM EDTA for 40 min at room temperature. All others were purchased in mature forms. Cells and tissue were extracted in lysis buffer (20 nM Tris–HCl at pH 7.5, 5 mM EGTA, 150 mM NaCl, 20 mM β-glycerol-phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.1% Tween-20) with 0.1 mM leupeptin freshly added to stabilize enzymes during electrophoresis and lysates were collected and cleared by centrifugation. Protein concentration was determined by micro BCA assay (Pierce). Non-reducing loading buffer (5X – 0.05% bromophenol blue, 10% SDS, 1.5 M Tris, 50% glycerol) was added to all samples prior to loading. Equal amounts of cell or tissue protein were resolved by 12.5% SDS–polyacrylamide gels containing 0.2% gelatin at 4 °C. Gels were removed and enzymes renatured in 65 mM Tris buffer, pH 7.4 with 20% glycerol for three washes, 10 min each. Gels were then incubated in activity buffer (0.1 M sodium phosphate buffer, pH 6.0, 1 mM EDTA, and 2 mM DTT freshly added) for 30 min at room temperature. For different pH conditions, 0.1 M sodium acetate buffers of pH 4, and sodium phosphate buffers of pH 6, 7, and 8 were used. Then this activity buffer was exchanged for fresh activity buffer of the same pH and incubated for 18–24 h (overnight) incubation at 37 °C. The gels were rinsed once with deionized water and incubated for 1 h in Coomassie stain (10% acetic acid, 25% isopropanol, 4.5% Coomassie Blue) followed by destaining (10% isopropanol and 10% acetic acid). Gels were imaged using an ImageQuant LAS 4000. For elastin zymography, 0.2% soluble elastin (Elastin products) was polymerized in the polyacrylamide gels in place of gelatin substrate.
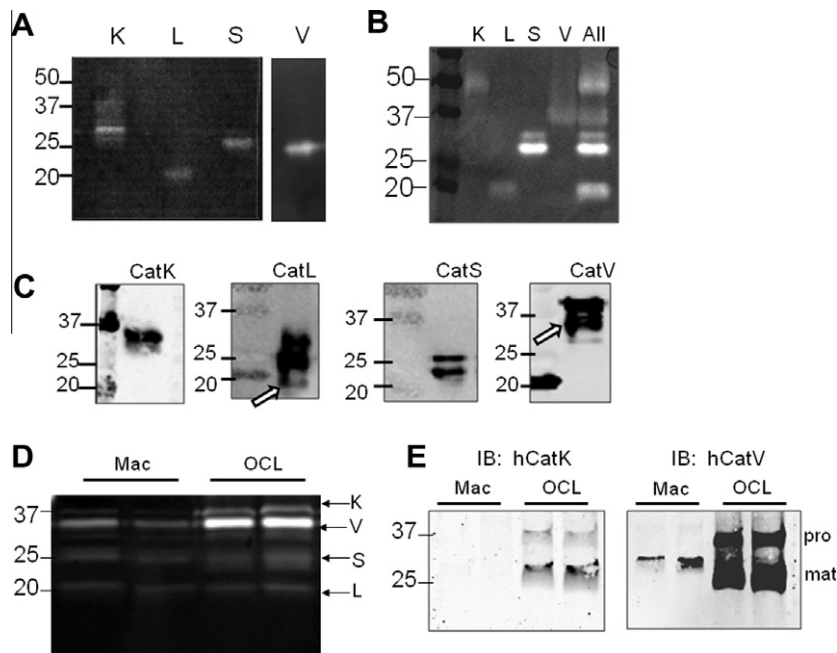
Western blots

SDS–PAGE was performed as described above without a gelatin or elastin substrate polymerized. Protein was transferred to a nitrocellulose membrane (Bio-Rad) and probed with monoclonal anti-human cathepsin K clone 182-12G5 (Millipore), anti-human cathepsin S and V antibodies (R&D Biosystems), or anti-mouse cathepsin L antibody (R&D Biosystems). Secondary donkey anti-mouse or anti-goat antibodies tagged with an infrared fluorophore (Rockland) were used to image protein with a Li-Cor Odyssey scanner.
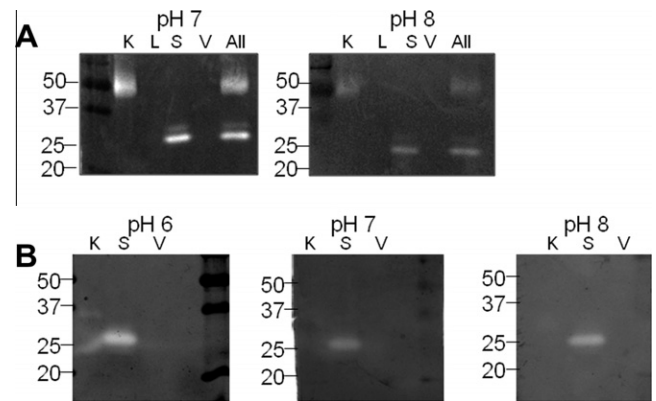
Results

Mature cathepsins K, L, S, and V activities can be detected by gelatin zymography

Recombinant cathepsins K and S from *E. coli,* cathepsin V with mutated glycosylation sites purified from *P. pastoris*, and cathepsin L isolated from human liver were loaded for zymography. Cathepsins K, S, and V are the nonglycosylated forms of the enzymes and cathepsin L contains both forms. All were active in the cathepsin zymography with bands at ~29 kDa for cathepsin K, ~21 kDa for cathepsin L, ~25 kDa for cathepsin S, and ~23 kDa for cathepsin V (Fig. 1A). To confirm the identity of the cathepsin bands, aliquots were loaded for Western blot, probed with their respective anti-cathepsin antibodies (Fig. 1C). Cathepsins L and V had multiple immunodetectable bands, but only one band was zymographically active under these conditions (open arrows).

To determine if glycosylation affects electrophoretic migration, recombinant cathepsins K, S, L, and V from eukaryotic expression systems were loaded for cathepsin zymography and a representative zymogram is shown in Fig. 1B. Cathepsin S maintained similar electrophoretic migration distances as that seen in Fig. 1A, but cathepsin K migration distance shifted from ~27 to ~50 kDa, and cathepsin V shifted from ~23 to ~37 kDa (Fig. 1B). All four glycosylated enzymes were loaded into the same well and similar migration distances were seen indicating that interaction between cathepsins in solution, as would occur in cellular extracts, does not alter their individual migration. Again, Western blotting confirmed the identity of the zymographically active bands and the shifts in electrophoretic migration distances (Fig. 1C). The open arrow indicates the zymographically active band of cathepsin V and cathepsin L on each respective blot. For all subsequent experiments, cathepsins from eukaryotic expression systems were used.

Fig. 2. Cathepsin zymography selectivity can be obtained through pH and substrate modifications. (A) Recombinant, glycosylated human cathepsins K, S, and V (10 ng) and cathepsin L (50 ng) were loaded separately and all in one lane for cathepsin zymography. The samples were incubated overnight in assay buffer, pH 7 or 8 prior to Coomassie staining. (B) 50 ng of human recombinant cathepsins K, S, and V were loaded for elastin zymography and incubated in pH 6, 7, or 8 assay buffers overnight. There was less cathepsin L and V signal at pH 7 and 8 and only cathepsin S maintained its activity in elastin zymography.

To test this assay on cellular extracts, we isolated monocytes from peripheral blood mononuclear cells (PBMCs) and differentiated them into macrophages and osteoclasts, two cell types that produce cathepsins, and osteoclasts specifically produce large amounts of cathepsin K under normal conditions [19,35,36]. Monocyte derived macrophages and osteoclasts were lysed and duplicates of 5 μg of protein from each were loaded for gelatin zymography. Bands (37, 25, and 20 kDa) were visible in both macrophages and osteoclasts, with the 25 and 20 kDa bands being consistent with cathepsins S and L, from the isolated enzyme studies,

Fig. 1. Mature cathepsins K, L, S, and V are zymographically active and migrate at distinct electrophoretic distances. (A) Recombinant cathepsins K, S, and V (1, 20, and 50 ng) from *E. coli* and cathepsin L (50 ng) isolated from human liver were loaded for cathepsin gelatin zymography and incubated overnight in acetate buffer, pH 6. The zymogram reveals zymographically active bands at different electrophoretic migration distances. (B) Mature, recombinant cathepsins K, S, and V (10 ng) from eukaryotic expression systems and cathepsin L (50 ng) isolated from human liver were loaded separately and all in one lane (where indicated) for gelatin zymography assayed at pH 6. (C) Western blot analysis of 50 ng of recombinant glycosylated cathepsin K, L, S, and V from eukaryotic expression systems also were loaded for non-reduced Western blotting. (D) Monocyte-derived macrophages and monocyte-derived osteoclasts were lysed and equal amounts of protein were loaded for cathepsin zymography and (E) reduced, fully denaturing Western blotting for cathepsins K and V. Procathepsin (pro) bands are at ~37 kDa and mature (mat) cathepsin bands are at ~27 kDa. Increased cathepsins K and V were detected in the osteoclasts compared to the macrophages.

respectively (Fig. 1D). The 35 kDa band was assumed to be cathepsin V to be consistent with Fig. 1B, but this band was brighter in osteoclasts and another band appeared just above it, around 37 kDa in the osteoclast lysates.

It is known that osteoclasts express cathepsin K specifically for bone resorption which supported the hypothesis that the upper band was osteoclast expression of cathepsin K. This was a different electrophoretic migration distance than that of the recombinant enzymes. To confirm this band was indeed cathepsin K, lysates from the monocyte derived macrophages and monocyte derived osteoclasts were loaded for reduced, denatured Western blotting, by adding β-mercaptoethanol to break disulfide bonds and boiling to fully denature and linearize the proteins. This differs from the zymography preparation, which maintains the disulfide bonds for proper refolding and renaturation, but does not fully linearize the peptide strand possibly resulting with a larger hydrodynamic radius which can affect their migration through the polyacrylamide gel during electrophoresis. Reducing Western blots probed with antibodies against human cathepsin K or V verified the identity of the increased cathepsin K in the osteoclasts by the visible detection of the pro- and mature forms of cathepsin K only in the osteoclast lysates where the upper 37 kDa band appeared. Both macrophages and osteoclasts had detectable levels of pro- and mature cathepsin V by Western blot, but a greater amount was present in osteoclasts, consistent with the brighter 37 kDa band in the zymography (Fig. 1E).

Cathepsin selectivity through pH and substrate modifications

Given the unexpected location of the cathepsin K band in monocyte derived osteoclasts and its close proximity to a brighter cathepsin V band, we wanted to modify the technique to uniquely distinguish each cathepsin. This would be useful for investigating new cell types or new disease conditions that might alter cathepsin activation or glycosylation. A new approach had to be employed to take advantage of unique cathepsin properties to enable selective distinction between cathepsins migrating at similar distances during the electrophoresis. A number of studies have indicated different substrate and pH-specific changes in proteolytic activity for different cathepsin family members [37,38] leading us to test the hypothesis that selectivity for cathepsin K, L, S, or V could be obtained by varying pH and substrate. Ten nano grams of cathepsins K, S, and V and 50 ng of cathepsin L were loaded for gelatin zymography, and 50 ng of each cathepsin were loaded for elastin zymography. These numbers were based on preliminary experiments to obtain visible bands. Gels were incubated in assay buffer at pH 6, 7, or 8 overnight prior to staining with Coomassie blue and visualization of cleared bands of proteolytic activity. Results are shown in Fig. 2.

Cathepsin K maintained activity on gelatin at pH 7 and 8, which contrasted significantly with cathepsins L and V activity (Fig. 1B and 2A) which were not seen at pH 7 or 8 on gelatin. Distinct bands for cathepsin K and S were at different electrophoretic distances and therefore, distinguishable from each other. Thus, a gelatin zymography at pH 7 would select for cathepsin K over V. Cathepsin S activity was stable under all pH conditions tested on both gelatin and elastin. With elastin zymography, cathepsins K and V activities were much weaker than that of cathepsin S (Fig. 2B) with a drastic reduction in their cleared band signals as pH increased such that at pH 7, cathepsins K and V retained little to no activity, and the assay conditions selected for cathepsin S (Fig. 2B).
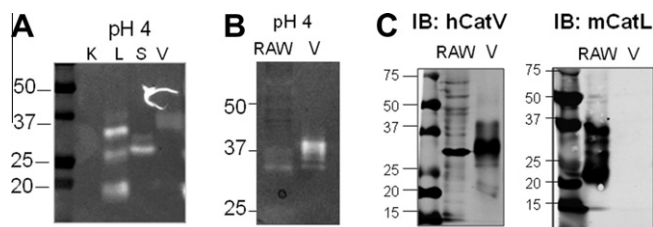
Selectivity for cathepsin V occurs at pH 4 after loss of cathepsin K band of activity

Unique conditions for cathepsin S had been determined, and cathepsin K had detectable proteolytic activity on gelatin at pH 7 where cathepsins L and V activity was attenuated (Fig. 2A), but conditions selective for cathepsin V had not yet been determined. An initial screen of RAW macrophage lysate displayed a strong band of activity at pH 4 that was neither cathepsin K nor S (data not shown), that we hypothesized was due to cathepsin V-like enzyme activity in macrophages. To test this, we loaded a gelatin zymogram with 10 ng of mature cathepsin K, S, and V, and 50 ng of cathepsin L, then incubated it in assay buffer, pH 4 (Fig. 3A). Cathepsin V band was detectable at ~37 kDa but the cathepsin K band was no longer active. Interestingly, multiple active bands of cathepsin L became visible at ~35, ~25, and ~20 kDa after incubation at pH 4, different from just the 20 kDa band detected at pH 6 (Fig. 1). Next, 5 μg of macrophage lysate and 50 ng recombinant cathepsin V were loaded for zymography and incubated in assay buffer, pH 6 and pH 4, prior to staining to confirm this band in RAW macrophages. Cathepsin V activity appeared at same distance as the cleared band of question in macrophage lysate (Fig. 3B).

RAW 264.7 cells are a mouse macrophage cell line, and murine cathepsin L is the homolog to human cathepsin V [17]. To confirm the identity of this band as murine cathepsin L, Western blotting was performed with 50 μg of macrophage lysate and probed with either an anti- human cathepsin V antibody or an anti-mouse cathepsin L antibody; mature, recombinant human cathepsin V was loaded as a positive control. Human cathepsin V and the immunodetected band in the macrophage lysate migrated similarly to ~37 kDa (Fig. 3C) corroborating the active band in macrophages was homologous to cathepsin V. The blot probed with anti-mouse cathepsin L detected the proform and the mature forms of mouse cathepsin L in the RAW 264.7 macrophages. Specificity of mouse cathepsin L antibody is shown by its inability to detect recombinant cathepsin V (Fig. 3C).

Selective zymography at pH 4 distinguishes the activity of cathepsin K from V in human cells and tissue from healthy and diseased conditions

Next goal was to apply this technology to natural changes in cells under healthy and diseased states and proper distinction of cathepsin K from V. Endothelial cells express cathepsin K at extremely low basal levels, but increase its expression under disturbed flow, inflammatory conditions, and atherosclerosis [13,14]. Human aortic endothelial cells (ECs) were grown to confluence and treated with or without 10 ng/mL tumor necrosis factor alpha (TNFα) for 20 h, after which lysates were collected for gelatin zymography. Stimulation of ECs with TNFα induced the 37 kDa cathepsin K band with detectable bands of cathepsins V and L for gelatin zymography at pH 6 (Fig. 4A). To select for cathepsin V, zymograms were

Fig. 3. Zymography selectivity for cathepsin V occurs at pH 4. (A) 10 ng of recombinant human cathepsins K, S, and V and 50 ng cathepsin L isolated from human liver were loaded for gelatin zymography and incubated overnight in assay buffer, pH 4. (B) Gelatin zymography performed with 5 μg of macrophage extract and 50 ng of recombinant cathepsin V were assayed at pH 4. The active bands at pH 4 in the RAW macrophage extract have similar electrophoretic distance as that of mature, human cathepsin V. (C) Western blot using anti-human cathepsin V antibodies and anti-mouse cathepsin L antibodies were used on 50 μg RAW 264.7 lysate and 50 ng of recombinant cathepsin V to confirm presence of each in the cell lysates.
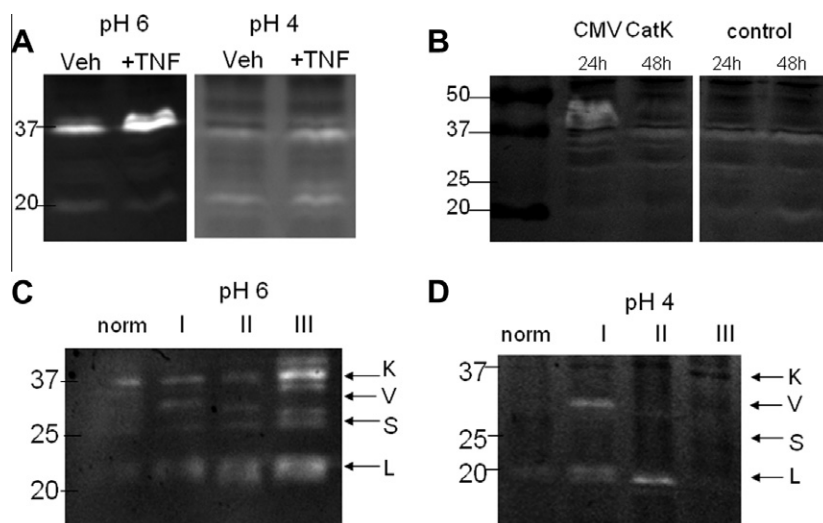
Fig. 4. Selective zymography at pH 4 distinguishes the activity of cathepsin K from V in human cells and tissue from healthy and diseased conditions. (A) ECs were cultured in the presence or absence of 10 ng/mL TNF for 24 h prior to lysis and loading for cathepsin zymography. Gels were incubated at pH 6 or pH 4 to select for cathepsin V over cathepsin K. Representative zymogram is shown. (B) ECs were transfected with cathepsin K gene on pCMVSport6 and cultured for 24 h or 48 h to overexpress cathepsin K. Zymogram at pH 6 is shown. (C) Lung tissue specimens from normal and from different stages of cancer progression were homogenized, and 10 µg of soluble total protein were loaded for gelatin zymography and incubated overnight in assay buffer, pH 6 and (D) pH 4 assay buffers. The bands of cathepsins K and S disappear from the gels leaving only cathepsins V (∼33/21 kDa) and L (21 kDa) bands.

incubated at pH 4, which diminished the 37 kDa band, but maintained detectable cathepsin V and cathepsin L bands of activity (Fig. 4A). Other ECs were transfected with CMVSport6 plasmid containing cathepsin K under the CMV promoter to drive constitutive overexpression and specifically corroborate the identity of the cathepsin K band. Lysates were collected 24 and 48 h after transfection, and equal protein amounts were loaded for zymography. In these cells, the 37 kDa band appeared, but did not in the control cells, corroborating its identity as cathepsin K (Fig. 4B).

To test the selective cathepsin V zymography on tissue, human lung tissue from normal and tumor specimens of different cancer stages (indicated by Roman numerals) were obtained and loaded for cathepsin gelatin zymography. Tumor specimens (II–IV) had greater cathepsin activity than the normal specimens and bands appeared at 37, 35, 25, and 21 kDa. To confirm the top band was cathepsin K and the 35 kDa band was cathepsin V, aliquots of the specimens were loaded for selective zymography, incubated at pH 4. No detectable active cathepsin bands in the presence of E64 indicated that bands are products of active cysteine proteases (data not shown). The 35 kDa band of interest remained in the lung specimens above the intensity of the other cathepsin signals confirming it as cathepsin V (Fig. 4D), cathepsin K (37 kDa) and S (25 kDa) bands were diminished, but lower molecular weight bands remained at the cathepsin L electrophoretic migration distance (∼21 kDa).

Discussion

This study has been able to show that cathepsins K, L, S, and V can all be detected by zymography in one cell lysate or tissue preparation under healthy and diseased states. Cathepsin K, L, S, and V activities were detected and distinguished in human endothelial cells, human monocyte derived macrophages and osteoclasts, murine macrophages, and normal and cancerous human lung tissue. Employing this assay with monocyte derived macrophages and monocyte derived osteoclasts were able to identify increased cathepsin V in osteoclasts compared to macrophages, and this is the first report to do so. Also, this assay was able to detect cathep-

sin V activity in human lung cancer in the absence of the pro-forms and other immunolabeled bands. It is not surprising that cathepsins V and L both have activity at pH 4, as they share 80% sequence homology, which may account for their renaturing ability and stability at the lower pH.

The ability to detect specific cathepsin activity in a complex cell or tissue lysate is critical in identifying the regulation of these proteases in healthy and diseased states; overdosing of cathepsin inhibitors due to improper quantification can block homeostatic functions of these enzymes and induce serious side effects [39]. We have also shown how different migration distances must be taken into account when interpreting results obtained from diseased states; although differential glycosylation exist for these enzymes, they seem to be active in zymography as shown with the recombinant cathepsins from prokaryotic and eukaryotic sources. It must also be considered that since the recombinant enzymes are from different sources, there is a possibility of different post-translational modifications. The apparent molecular weight and electrophoretic migration distances of cathepsins are different, partially due to glycosylation and secretion mechanisms. As an example, cathepsin V has been shown to have two putative N-glycosylation sites on the mature enzyme and when these sugars are cleaved the migration of the enzyme is altered by 4–7 kDa [3]. Improper glycosylation mistargets these cathepsins to other cellular compartments or for secretion [23,40,41], which occurs during pathological overexpression of cathepsins.

Cathepsin zymography has multiple advantages over other methods. Individual cathepsin activity can be visually confirmed by band location and intensity can be quantified with densitometric analysis. This is an advantage over fluorescent reporter substrate assays that, when cleaved, release aminomethylcoumarin or other quenched fluorescent motifs. Enzyme concentrations, reporter substrate concentrations, and cross-reactivity with reporter substrates, all add confusion to accurate interpretation of the total fluorescent signal. Serine proteases, matrix metalloproteinases, and other enzyme families also contribute to hydrolysis of these reporter substrates confounding proteolytic readout and attribution of that activity to a particular enzyme.

The zymography method described here overcomes these challenges by (a) incubating gels in acidic conditions to drastically reduce activity of MMPs and serine proteases, (b) allowing the addition of inhibitors that block enzymatic activity of other proteases, (c) selecting for enzymes capable of thermodynamically favorable renaturation after non-reducing, partial denaturation by SDS and (d) exploitation of unique enzyme structural properties (size, glycosylation, disulfide bridge number and locations) that impart distinct electrophoretic migration distances that may not be seen in reduced, fully linearized SDS–PAGE and immunoblotting. As an added benefit, this assay does not require antibodies which expands its use to different species as cathepsin structures are fairly well conserved; here we have used mouse and human specimens. This also reduces costs compared to immunobased methods such as ELISA, Western blotting, and immunohistochemistry, and remove concerns of nonspecific antibody binding and pro-cathepsin detection interference.

Limits of detection for each enzyme at each pH and substrate will need to be determined. We have already determined that cathepsin K can be detected as low as 0.1 ng at pH 6 [34] and 0.8 ng at pH 7 (data not shown), but cathepsins L, S, and V limits are higher and required a greater amount of enzyme to elicit a detectable signal after Coomassie staining (Fig. 1). Fair comparisons of cathepsin activity of different samples loaded in the same gel can be made, but absolute standards can also be loaded to fit the quantified densitometry signal and calculate an actual value to compare across different gels [34,42].

Conclusions

Many types of cancers and tissue destructive diseases are caused by altered cathepsin activity and regulation. Understanding and detecting this cathepsin mediated tissue remodeling is important not only for basic science research, but also for clinical purposes. Broad application of this cathepsin K, L, S, and V multiplex zymography will provide a medium throughput and inexpensive protocol with widespread utility. Tools that add greater selectivity such as the zymography protocols and modifications presented here, that enable proper cathepsin identification, and quantification of those signals will assist appropriate pharmaceutical inhibitor dosing and the determination of pathological functions due to upregulated cathepsins for future investigations and, hopefully, answer previously unsolved questions of proteolytic activity.

Acknowledgments

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