Atherosclerosis is characterized by a thickening and loss of elasticity of the arterial wall. Loss of elasticity has been attributed to the degradation of the arterial elastin matrix. Cathepsins K and S are papain-like cysteine proteases with known elastolytic activities, and both enzymes have been identified in macrophages present in plaque areas of diseased blood vessels. Here we demonstrate that macrophages express a third elastolytic cysteine protease, cathepsin V, which exhibits the most potent elastase activity yet described among human proteases and that cathepsin V is present in atherosclerotic plaque specimens. Approximately 60% of the total elastolytic activity of macrophages can be attributed to cysteine proteases with cathepsins V, K, and S contributing equally. From this 60%, two-thirds occur extracellularly and one-third intracellularly with the latter credited to cathepsin V. Ubiquitously expressed glycosaminoglycans (GAGs) such as chondroitin sulfate specifically inhibit the elastolytic activities of cathepsins V and K via the formation of specific cathepsin-GAG complexes. In contrast, cathepsin S, which does not form complexes with chondroitin sulfate is not inhibited; thus suggesting a specific regulation of elastolytic activities of cathepsins by GAGs. Because the GAG content is reduced in atherosclerotic plaques, an increase of cathepsin V activity may accelerate the destruction of the elastin matrix in diseased arteries.

Atherosclerosis is characterized by arterial intimal enlargement and subsequent lipid deposition leading to the formation of blood stream obstructing plaques. The infiltration of monocyte-derived macrophages (MDMs) and smooth muscle cells (SMCs) into the intima of the inflamed artery contributes to the formation of atherosclerotic lesions. Atherosclerotic lesions resident MDMs and SMCs produce a large number of extracellular matrix-degrading enzymes (1), such as cysteine proteases (2–5) and matrix metalloproteinases (MMPs) (6–8). Elastin and collagen are the two major extracellular matrix components that provide elasticity and tensile strength to the arterial wall. The destruction of elastin and collagen causes a weakening and rupture of blood vessels (9, 10). MMPs, serine, and cysteine proteases have been identified as major elastolytic proteases in arteries (11, 12). Among cysteine proteases, cathepsins S and K have been considered as the most potent elastolytic activities with cathepsin K exhibiting a slightly higher activity than cathepsin S (13, 14). However, cathepsin K-deficient human macrophages derived from patients with pycnodysostosis were shown to retain their high cysteine protease-dependent elastolytic activities (15). This finding implied that additional cathepsins may contribute to the overall elastolytic activity in human macrophages.

We and others (16–18) have identified and partially characterized a novel human cysteine protease closely related to cathepsin L that was named cathepsin V (also known as cathepsin L2). Cathepsin V shares 80% protein sequence identity with cathepsin L, but in contrast to the ubiquitously expressed cathepsin L, its expression is mostly confined to the thymus and testis. Recently, we found that recombinant cathepsin V has the ability to efficiently convert MHC/class II-associated invariant chain Ii into CLIP, suggesting that cathepsin V is the protease that controls the generation of antigen-presentable αβ-CLIP complexes in the human thymus (19). No human cathepsin L was found in thymic antigen presenting cells, thus contrasting findings from studies using cathepsin L-deficient mice, which established a critical role for cathepsin L in thymic antigen presentation (20). Our data supported the hypothesis that human cathepsin V, but not human cathepsin L, is the functional equivalent of mouse cathepsin L (16, 19). Despite significant efforts to identify a mouse orthologue of human cathepsin V with a thymus-specific expression pattern, this protease has not been found.

In this study, we determined the expression of cysteine-dependent cathepsins in atheroma tissue and MDMs and characterized their elastolytic activities. Besides the known elastolytic activities of cathepsins S and K in MDMs, we describe for the first time the expression of cathepsin V in those cells and demonstrate that this protease exhibits the highest elastolytic activity yet described among mammalian proteases and that it mostly contributes to the intracellular degradation of elastin. Moreover, we demonstrate, that the elastolytic activities of cathepsins are regulated by biologically relevant glycosaminoglycans.
The pGEMT vector (Promega, Madison, WI), digested with NdeI/BamHI, cDNA using the forward primer 5’-AGAATCTCCTAGCCCCACA-3’ and the reverse primer 5’-AGGAGGAGCAAT-GATCCTCTTGAT-3’. The purification procedure was modified for the pET16b vector (Novagen, Madison, WI). The sequence of the final plasmid was confirmed by automated DNA sequencing. BL21(DE3) cells transformed with MPP-12/pET16b were grown at 37 °C to an OD600 of 1 and then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 3 h. Cells were harvested by centrifugation and treated with lysozyme (1 mg/ml) in lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100) and sonicated on ice. The washed pellet was resuspended in denaturing buffer (100 mM NaH2PO4, pH 8.0, 10 mM Tris, 8 M urea) and incubated at room temperature for 30 min by gentle vortexing. After centrifugation, the supernatant was applied on Ni-NTA resin equilibrated with the same buffer and incubated at room temperature for 1 h. Unbound proteins were washed out with washing buffer (50 mM NaH2PO4, pH 4.5, 10 mM Tris, 8 M urea) and eluted with elution buffer (100 mM NaH2PO4, pH 4.5, 10 mM Tris, 8 M urea) and diluted into folding buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 5 mM CaCl2, 50 μM ZnSO4, 0.05% Brij 35, 2.5 mM 1-t-glutathione-oxidized, 2.5 mM 1-t-glutathione-reduced, and 20% glycerol) to a final concentration of 4 mM urea. The refolding reaction was carried out at 4 °C overnight. The urea concentration was reduced to 2, 1, 0.5 and 0 μM by dialysis against dialysis buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 5 mM CaCl2, 0.05% Brij35). Protein concentrations were determined using the Bradford assay.

**Western Blot Analysis**—Atheroma tissue was kindly provided by Dr. Marin from the Mount Sinai Hospital, New York, NY. The tissue was suspended in lysis buffer (100 mM Tris-HCl, pH 7.2, 1 mg/ml proteinase K, and 1 mM EDTA) as previously described (19, 30) with some modifications. Briefly, lysosomal fractions of cysteine proteinases were obtained using the ECL system. The specificity of the mAb was confirmed by epitope mapping and detection of recombinant human cathepsin V by immunoblotting. The antibody showed no cross-reactivity with human cathepsins L, H, S, K, and W in ELISA and immunoblotting (data not shown). PabSasha and ma533/1 have been previously characterized (19, 28, 29).

**Active Site Labeling of Cysteine Proteinases**—Active site labeling experiments of cysteine proteinases were performed as previously described (19, 30) with some modifications. Briefly, proteins from MDMs were incubated with or without Mu-Leu-Hph-VS-Ph (1 nM, 100 mM Tris-HCl, pH 7.4) and 2 mM EDTA using a 26-gauge needle. Cellular lysates were centrifuged at 15,000 g for 10 min and the resulting supernatant was centrifuged at 20,000 g for 20 min. The pellet was resuspended in 50 mM sodium acetate buffer (pH 5.5) containing 2.5 mM DTT/EDTA and incubated with inhibitors.

**Combinant Human Cathepsins**—Human cathepsin V was expressed in Pichia pastoris using the expression vector pPIC-9K (Invitrogen, Carlsbad, CA) as previously described (19, 30). Human cathepsins L, K, and F were expressed using the same expression system (22, 23). Human cathepsin S was produced in Sf9 cells with the help of the baculovirus expression system (24). Recombinant human mature cathepsin B was kindly provided by Dr. Robert Menard (Biotechnology Research Institute, Montreal, Quebec, Canada). Cathepsins V, L, K, and S were activated by treatment with pepsin and purified on Sepharose (Amersham Biosciences) as previously reported (14, 16, 25). The active site concentrations of the purified cathepsins V, L, K, and S were determined by E-64 titration (26).

**Expression and Purification of Human Recombinant Metalloelastase (MMP-12) Catalytic Domain**—The purification procedure was modified from that of Parkin et al. (27). DNA encoding the human MMP-12 catalytic domain was prepared by PCR cloning from human placenta cDNA using the forward primer 5’-GATCCTCATATGGTAGGGAAT-3’ and the reverse primer 5’-GGATCCCTTTATCCAGGAGATCTTGAT-3’. The resulting 500-bp PCR product was subcloned into the pGEMT vector (Promega, Madison, WI), digested with Ndel/BamHI and cloned into the Ndel/BamHI sites of the pET16b vector (Novagen, Madison, WI). The sequence of the final plasmid was confirmed by automated DNA sequencing. BL21(DE3) cells transformed with MMP-12/pET16b were grown at 37 °C to an OD600 of 1 and then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 3 h. Cells were harvested by centrifugation and treated with lysozyme (1 mg/ml) in lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100) and sonicated on ice. The washed pellet was resuspended in denaturing buffer (100 mM NaH2PO4, pH 8.0, 10 mM Tris, 8 M urea) and incubated at room temperature for 30 min by gentle vortexing. After centrifugation, the supernatant was applied on Ni-NTA resin equilibrated with the same buffer and incubated at room temperature for 1 h. Unbound proteins were washed out with washing buffer (50 mM NaH2PO4, pH 4.5, 10 mM Tris, 8 M urea) and eluted into folding buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 5 mM CaCl2, 50 μM ZnSO4, 0.05% Brij 35, 2.5 mM 1-t-glutathione-oxidized, 2.5 mM 1-t-glutathione-reduced, and 20% glycerol) to a final concentration of 4 mM urea. The refolding reaction was carried out at 4 °C overnight. The urea concentration was reduced to 2, 1, 0.5 and 0 μM by dialysis against dialysis buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 5 mM CaCl2, 0.05% Brij35). Protein concentrations were determined using the Bradford assay.

**Elastolytic Activity of Cathepsin V**

The elastolytic activity of the recombinant human cathepsin V was measured by the method of Parkin et al. (34). Briefly, MDMs (2 × 106) were homogenized in 0.25 M sarcosine containing 25 mM HEPES (pH 7.4) and 100 mM DTT/EDTA using a 26-gauge needle. Cellular lysates were centrifuged at 2000 g for 2 min, and the resulting supernatant was centrifuged at 15,000 g for 20 min. The pellet was resuspended in 50 mM sodium acetate buffer (pH 5.5) containing 2.5 mM DTT/EDTA and incubated with inhibitors.

**Combinant Human Cathepsins V, K, and S**—Combinant human cathepsins V, K and S (0.3 ng each) were incubated with 1 nM and 10 nM Mu-Leu-Hph-VS-Ph or with 10 nM Mu-Np2-Hph-VS-2Np, respectively, in 10 μl of 0.1 M sodium acetate buffer (pH 5.5) containing 0.5% bovine serum albumin, 2.5 mM DTT/EDTA at 37 °C for increasing time periods (0, 5, 30, 60 min) prior to the incubation with DCG-04. The reactions were stopped by the addition of SDS sample buffer. The reaction mixtures were separated on 10% Tris-glycine gels by SDS-PAGE and transferred on polyvinylidine difluoride membranes. After blocking with 1% bovine serum albumin/T-PBS, membranes were probed with streptavidin-horseradish peroxidase at room temperature for 1 h. DCG-04 inhibited proteases were visualized using the ECL system.
**Elastin Congo Red Assay**—The elastolytic activity of each enzyme was tested by a modification of the procedures of Rust et al. (35, 36). 10 mg of Congo Red elatin (Sigma) were suspended in 0.9 ml of 50 mM sodium acetate buffer (pH 5.5) containing 2.5 mM DTT and 2.5 mM EDTA (for cysteine protease), or 50 mM Tris-HCl (pH 7.2) with or without 5 mM CaCl₂ (for metalloproteinases and serine proteases, respectively). The digest reaction was initiated by the addition of the enzymes (final concentration of 1 μM) and then incubated at 37 °C for 18 h. Reactions were carried out in the absence or presence of 0.15% C4-S, C6-S, DS, or heparin (Sigma). Finally, the reaction mixtures were centrifuged at 10,000 × g for 15 min, and the OD₄₉₀ of the supernatants was recorded.

In Situ Assay Ames—To determine whether various inhibitors used to block the elastolytic activity of MDMs penetrate into the cells, the intracellular hydrolysis of a synthetic peptide substrate for cathepsin B (Z-Arg-Arg-4-MjNA, Bachem Inc., Bubendorf, Switzerland) was monitored. The assay using Z-Arg-Arg-4-MjNA was performed as previously described (37). Briefly, 12-day cultures of MDM were incubated for 24 h with either 1 μM Mu-Leu-Hph-VS-Ph, 1 μM Mu-Np2-Hph-VS-2Np, 1 μM E-64d, 1 μM E-64, or 100 μM E-64. Control experiments did not contain an inhibitory compound. Inhibitor treated or untreated cells were subsequently incubated with a mixture of 1 mg/ml of Z-Arg-MjNA, 1 μM 5-nitrosalicylaldehyde, 1 mM DTT, 2.7 mM l-cysteine, and 0.25 mM EDTA in 100 mM sodium acetate buffer (pH 5.5). Protease released 4-methoxy-7-H₉₂₅₃ labeled cathepsins were preincubated in 100 mM sodium acetate buffer, pH 5.5 containing 2.5 mM DTT and 2.5 mM sodium acetate buffer (pH 5.5) containing 2.5 mM DTT and 2.5 mM sodium acetate buffer (pH 5.5). The fluorescence signal was detected by fluorescent microscopy using a Nikon Eclipse E800 microscope (excitation 440–500 nm and emission above 510 nm). To determine whether insoluble elastin particles are taken up by cells, MDMs were seeded on the coverslips in a 6-well plate and allowed to attach overnight. 2 mg of rhodamine-conjugated elastin (Calbiochem, San Diego, CA) suspension in culture media per well were added and after 3 days of incubation, cells were washed three times with ice-cold PBS (pH 7.4). Then cells were mounted using Fluoromount-G (Southern Biotechnology Association Inc., Birmingham, AL), and the fluorescent signal was detected by fluorescent microscopy using a Nikon Eclipse E800 microscope (emission 570 nm).

**H-labeled Elastin Degradation**—H-labeled elastin was kindly provided by Dr. Stephen J. Weiss (University of Michigan, Ann Arbor, MI). Elastin-coated plates were prepared by drying 2 mg of H-labeled elastin on the entire surface of each well of 12-well plates. 12-day MDMs prepared from healthy donors were added and cultured in the presence or the absence of inhibitors, Mu-Leu-Hph-VS-Ph, Mu-Np2-Hph-VS-2Np, or Mu-Leu-Hph-VS-Ph plus GM6001 (Calbiochem), E-64 or E-64d at concentrations as indicated in Figs. 5 and 6. After 24 h of incubation at 37 °C, supernatants were collected, centrifuged at 10,000 × g for 10 min, and solubilized elastin was quantified by scintillation counting. Results are expressed as relative elastin degradative activities (in percent).

**Substrate Assay using Z-Phe-Arg-MCA**—Steady state kinetics were performed using the fluorogenic dipeptide substrate, Z-Phe-Arg-MCA as previously described (14). The enzymatic activity was followed by monitoring the release of the fluorogenic leaving group, MCA, at an excitation wavelength of 380 nm and an emission wavelength of 450 nm using the Molecular Devices SpectraMax Gemini spectromicrofluroimeter. The Kₘ and Vₘ values were determined by nonlinear regression analysis. Cathepsin V activities were assayed at 25 °C in the absence or presence of C4-S and 300 mM NaCl at a fixed enzyme concentration (1 μM) and variable substrate concentrations (1 to 10 μM) in 100 mM sodium acetate buffer (pH 5.5) containing 2.5 mM DTT and 2.5 mM EDTA.

**Labeling of Cathepsins and Electrophoretic Mobility Shift Assay**—DCG-04, an epoxide inhibitor derivative of E-64 containing a tyrosine residue for iodination was used to label active cathepsins. The sample was iodinated as described previously (38). 50 ng of [¹²⁵I]DCG-04 labeled cathepsins were preincubated in 100 mM sodium acetate buffer, pH 5.0 containing 1 mM DTT/EDTA in the presence or the absence of 0.1% C4-S, respectively, for 30 min and then mixed at 37 °C with 20% non-reducing protein loading buffer and preheated agarose gel. The sample was loaded into the well of a 0.5% agarose gel and separated at 50 V, 250 mA for 30 min in a running buffer containing 125 mM sodium acetate, pH 5.0, 0.5 mM EDTA, 1 mM DTT, 40 mM NaCl, and 0.1% CHAPS. After gel drying, [¹²⁵I]DCG-04 labeled cathepsins were visualized on x-ray film.

**Steady state kinetics** were performed using the fluorogenic dipeptide substrate, Z-Phe-Arg-MCA as previously described (14). The enzymatic activity was followed by monitoring the release of the fluorogenic leaving group, MCA, at an excitation wavelength of 380 nm and an emission wavelength of 450 nm using the Molecular Devices SpectraMax Gemini spectromicrofluroimeter. The kₘ and Vₘ values were determined by nonlinear regression analysis. Cathepsin V activities were assayed at 25 °C in the absence or presence of C4-S and 300 mM NaCl at a fixed enzyme concentration (1 μM) and variable substrate concentrations (1 to 10 μM) in 100 mM sodium acetate buffer (pH 5.5) containing 2.5 mM DTT and 2.5 mM EDTA.

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**RESULTS**

**Expression of Cathepsins in Human Atheroma Tissue**—Using specific antibodies, we determined the expression of cathepsin polypeptides in human atheroma by Western blot analysis. As shown in Fig. 1, the extract of human atheroma tissue obtained from a patient with atherosclerosis contained the precursor (42 kDa) and mature forms (28 kDa) of cathepsin K and mature forms of cathepsins S and L as previously described. Interestingly, immunoreactive bands with apparent molecular masses of 43 and 29 kDa for a novel tissue-specific cysteine proteinase, cathepsin V, were also detected. Cathepsin V protein has been previously described only in thymus (19) and cornea (39), and on the mRNA level in thymus, testis, cornea, and a colon tumor cell line (16–18).

**Expression of Cathepsin Transcripts in Monocytes and MDMs**—Human macrophages have been shown to differentially express mRNAs of several gene products such as osteopontin, GP-39, and cathepsin K (15, 40) depending on their stage of activation. To determine the level of cathepsin mRNA, and in particular that of cathepsin V expression in monocytes and macrophages, monocytes were cultured with 200 units/ml IL-3 for 0, 6, and 12 days to differentiate them into macrophages. Interleukin-3 treatment led to macrophage–typical morphological changes of monocytes, and to an increase in mRNA expression of the macrophage markers, osteopontin and GP-39. We also confirmed the expression of the macrophage marker, CD68, by immunofluorescence microscopy (data not shown). MDMs markedly increased the expression of all cathepsin mRNAs tested during differentiation, however, the expression pattern of cathepsins V and K were different from that of cathepsins S and L. Whereas cathepsins S and L displayed a constitutive expression pattern during differentiation, cathepsin K and cathepsin V where induced only after 6 or 12 days, respectively (Fig. 2A). Real-Time PCR analysis confirmed these results and showed that cathepsins S and L expression each increased by only 60% during the differentiation process. In contrast, cathepsins K and V increased their expression levels 7–27-fold, respectively. However, the level of mRNA expression for cathepsin S in macrophages was at least 4× higher than that of cathepsin V, 2.5× higher than that of cathepsin L, and ~30× higher than that of cathepsin K (Fig. 2B).

**Comparison of Elastolytic Activities Among Cathepsins, Elastases, and MMPs**—We determined the time course of the elastolytic activities of human cathepsins V, L, and K using Congo Red elatin as substrate and measured the residual
activities of these samples with the fluorogenic substrate, Z-Phe-Arg-MCA. Although the enzyme stabilities of cathepsins V and L were very similar at 37 °C and pH 5.5 (Fig. 3B), only cathepsin V displayed a strong elastolytic activity (Fig. 3A).

Consistent with previous data, cathepsin K was less stable under these conditions and thus exhibited a lower cumulative activity when compared with cathepsin V.

Next, we compared the elastolytic activity of cathepsin V with those of various other representative elastolytic enzymes. Surprisingly, the elastolytic activity of cathepsin V was 2–8-fold higher when compared with that of other known elastolytic activities such as pancreatic and leukocyte elastases, MMP-2 and MMP-12, and cathepsins K and S, respectively (Fig. 4).

Human cathepsin L displayed only a negligible activity toward Congo Red elastin. Interestingly, mouse cathepsin L revealed a comparably high activity as human cathepsin K. This is further corroborating our hypothesis that mouse cathepsin L represents the functional equivalent of human cathepsin V and not that of human cathepsin L. The low elastolytic activity of human cathepsin L is in accordance with previous studies using the recombinant protease form but is in contrast to a report showing a significant elastase activity of native human cathepsin L (41). The activities of both forms toward synthetic cysteine protease substrates, however, are very similar. It only can be speculated why there appears to be a difference between the native and recombinant forms of cathepsin L with regard to their elastolytic activities. It could be a difference in their glycosylation pattern or a contamination of the native enzyme. Protease contaminations of the recombinant form can be excluded, since 1) the Pichia expression system used does not express any papain-like cysteine protease (25) and 2) its elastolytic activity could be completely abolished with the general cysteine protease inhibitor, E64, thus excluding any non-cysteine protease related activities (not shown). Altogether, these findings suggest that cathepsin V represents the most potent mammalian elastolytic enzyme yet described.

Identification of Cathepsins in Macrophages and Selective Inhibition of [3H]Elastin Degradation—DCG-04 was used to identify cathepsins expressed in MDMs by active-site labeling. The labeling of cathepsins by DCG-04 in MDM lysosomal extracts was completely prevented in the presence of 1 μM Mu-Leu-Hph-VS-Ph (Fig. 5B). Using recombinant human elastolytic cathepsins, Mu-Leu-Hph-VS-Ph at 1 μM abolished the labeling of cathepsins V, K, and S by DCG-04 (Fig. 5A). In contrast, Mu-Leu-Hph-VS-Ph at very low concentrations revealed a significant specificity for individual cathepsins. As previously shown, Mu-Leu-Hph-VS-Ph (also known as LHVS) specifically inhibited cathepsin S at very low concentrations (5 nM) (31). Here we show that at 1 nM concentration of Mu-Leu-
Hph-VS-Ph, cathepsins V and K are still active and thus could be labeled by DCG-04 whereas the activity of cathepsin S was completely blocked. Mu-Np2-Hph-VS-2Np, a derivative of Mu-Leu-Hph-VS-Ph where the leucine and the vinyl sulfone benzene group has been replaced by naphthylalanine and vinyl sulfone naphthene, is a potent inhibitor at low concentration for most cathepsins except cathepsin K (32). At 10 nM, Mu-Np2-Hph-VS-2Np inhibited cathepsins S and V but not cathepsin K (Fig. 5A). DCG-04 labeling of lysosomal extracts prepared from MDM resulted in three bands, which represented cathepsin B (upper band), cathepsins S and V (middle band), and cathepsin K (lower band) (Fig. 5B). The identity of the appropriate bands was deduced from corresponding human recombinant cathepsins labeled with DCG-04 (Fig. 5B, ctrl) and by immunoblot analysis of the MDM preparations (data not shown). Treatment of the lysosomes with 1 nM of Mu-Leu-Hph-VS-Ph led to a decrease of the intensity of the middle band consistent with a specific inhibition of cathepsin S. The remaining band, therefore, must represent cathepsin V. Cathepsins K and B were not affected by the inhibitor at this low concentration. In contrast, 1 μM Mu-Leu-Hph-VS-Ph inhibited all cathepsins present in the lysosomal extract. 10 nM of Mu-Np2-Hph-VS-2Np completely inhibited cathepsins S and V and to lesser degree cathepsin B but left cathepsin K mostly untouched. As expected,
CA-074, a selective cathepsin B inhibitor, prevented the DCG-04 labeling of the upper band (Fig. 5B).

MDMs were grown for 24 h at 37 °C in the presence of [3H]elastin with and without protease inhibitors. Using both inhibitors, Mu-Leu-Hph-VS-Ph and Mu-Np2-Hph-VS-2Np, at selective concentrations permitted the estimation of the individual contributions of all three elastolytic cathepsins. Mu-Leu-Hph-VS-Ph and Mu-Np2-Hph-VS-2Np at the non-selective concentration of 1 μM reduced the hydrolysis rate by approximately 60%, thus representing the total contribution of cathepsins to intra- and extracellular elastin degradation. 1 μM E-64d inhibited the intracellular activities, 1 μM E-64 inhibited the extracellular cathepsin activities and 100 μM E-64 inhibited both, extra- and intracellular activities.

For example, the vinyl sulfone inhibitor, Mu-Np2-Hph-VS-2Np, displays a second order rate constant of inactivation for cathepsin S, which is more than 10^5× higher than that for cathepsin K (mostly caused by their differences in the appropriate K_i values) (32). Thus, at an inhibitor concentration which is in the range of the K_i for one enzyme but several orders of magnitude below the one of a related enzyme an inhibition assay will allow discrimination between both enzymes if the exposure time to the inhibitor is not infinite. On the contrary, if incubation time and inhibitor concentration are sufficiently high, all suitable enzymes will be inhibited.

These experiments indicate that ~20% of the total elastolytic activity present in MDMs can be attributed to cathepsin S, 20% to cathepsin K, and 20% to cathepsin V. The remaining 40% are cysteine proteinase independent and can be partially assigned to matrix metalloproteinases. GM6001, a general MMP inhibitor, reduced the elastolytic activity of MDMs by an additional 25–30% leaving ~10–15% of the total hydrolysis rate to non-cysteine and non-MMP activities.

Mu-Leu-Hph-VS-Ph and Mu-Np2-Hph-VS-2Np are both cell permeable inhibitors and do not permit the discrimination between extra- and intracellular elastin degradation. Both inhibitors reduced the elastolytic activity of MDMs by 60% (Fig. 6A). Thus, we employed E-64 (non-cell permeable) and E-64d (cell permeable) as cysteine protease inhibitors to address the question whether cathepsin-mediated elastin degradation by MDM happens primarily intra- or extracellularly. E-64d is a cell permeable inactive ethyl ester derivative of E-64, which requires the cleavage of the ester bond by intracellular esterases for activation. Thus, E-64d mediated inhibition should strictly represent the blockade of intracellular cathepsin activities. Conforming with the expected intracellular activity of E-64d and the extracellular activity of E-64, 1 μM E-64d completely inhibited the intracellular hydrolysis of the cathepsin B substrate, Z-Arg-Arg-Mca (Fig. 7D) whereas the same concentration of E-64 had no effect (Fig. 6A). In the absence of cysteine protease inhibitors, cells hydrolyze Z-Arg-Arg-MCA and produce a fluorescent signal within the lysosomal compartment (Fig. 7A). Using insoluble [3H]elastin as substrate, we could demonstrate that 1 μM E-64 reduced total elastin degradation by ~20%, whereas 1 μM E-64 inhibited about 40–45% of the elastolytic activity (Fig. 6B). These data can be interpreted that ~20% of the released tritiated elastin degradation products were derived from intracellular and 40–45% from extracellular degradation. To demonstrate that insoluble elastin is phagocytosed by MDMs, we incubated the macrophages in the presence of rhodamine-labeled elastin. Here, the MDMs revealed intracellular vesicular red fluorescence signals, which suggest the uptake of labeled elastin particles (Fig. 7G and H).

Very high concentrations of E-64 (100 μM as recently used in studies by Punturieri et al. (15), the inhibitor also blocked completely the intracellular hydrolysis of Z-Arg-Arg-McaNA indicating an uptake of the inhibitor by an endocytotic pathway (Fig. 7F). The 60% reduction of elastolysis in the presence of 100 μM E-64 (Fig. 6B) identical to the inhibition observed for the vinyl sulfone inhibitors; Fig. 6A) therefore represents the inhibition of the intra- as well as extracellular activities of MDMs. As seen in Fig. 7, B and C, both vinyl sulfone inhibitors were cell permeable and completely blocked the hydrolysis of the fluorogenic substrate.

Effect of GAGs on the Elastolytic Activities and Complex Formation of Cathepsins with C4-S—GAGs are known to provide resilience to the alveolar wall and to possess inhibitory activity toward leukocyte elastase (42). Recently, we demonstrated that GAGs might modulate the collagenase activities of cathepsins (43, 44). To determine whether GAGs can affect the
elastolytic activity of proteases, biologically relevant GAGs such as C4-S, DS, C6-S, and heparin were incubated with elastolytic proteases, and their activities toward synthetic peptide substrates and insoluble elastin were determined. The elastolytic activities of cathepsins V and K were inhibited in the presence of 0.15% GAGs by 60–90% (Fig. 8A). Heparin had the highest inhibitory effect against cathepsins among the tested GAGs and also effectively inhibited serine proteases (Fig. 8B) as previously documented (42). On the other hand, with the exception of heparin, other GAGs had little or no effect on the elastolytic activities of cathepsin S, pancreatic elastase, leukocyte elastase, MMP-2, and MMP-12 (Fig. 8, A and B). Furthermore, Fig. 8C revealed that C4-S suppressed the elastolytic activities of cathepsins V and K in a dose-dependent manner with 80% inhibition at a concentration of 0.3% (w/v). In contrast, the activity of cathepsin S was only mildly reduced by 20% at high concentration of C-4S. These results indicated that C4-S specifically inhibited cathepsins V and K rather than cathepsin S.

To elucidate the selective inhibition of elastolytic activities by C4-S in more detail, we investigated whether cathepsin V is capable to form a complex with C4-S similar to that described for cathepsin K (43). Complex formation between cathepsins and C4-S was analyzed by the electrophoretic mobility shift assay. Cathepsins K and V formed complexes in the presence of C4-S and, thus migrated further to the anode than the enzymes in the absence of C4-S (Fig. 9). In contrast, cathepsin S migrated identically in the presence or absence of C4-S suggesting the lack of complex formation.

High salt concentrations (>200 mM) lead to a dissociation of cathepsin/C4-S complexes as previously shown for cathepsin K (43) but did not result into a restoration of the elastolytic activities of the proteases. NaCl inhibited the complex-forming cathepsins K and V as well as cathepsin S which lacked complex formation (Fig. 10). The inhibition of the elastolytic activities of all three cathepsins suggested the interference of protease-substrate (elastin) interactions at high salt concentrations. In the presence of both, C4-S and NaCl, an additive inhibitory
The effect was observed for cathepsin K but not for cathepsin S (the elastolytic activity of cathepsin V was completely suppressed in the presence of NaCl). To examine, whether C4-S and/or NaCl affected the catalytic activity of cathepsin V, we determined the kinetic parameters for the cathepsin V-catalyzed hydrolysis of the synthetic fluorogenic substrate, Z-Phe-Arg-MCA, in the absence and presence of C4-S and NaCl, respectively (Table I).

Both C4-S and NaCl had no effect on the appropriate $K_m$ and $k_{cat}$ values suggesting that the inhibitory effect of both components was not associated with the blocking or alteration of the active site by itself. Ionic and hydrophobic interaction might be necessary for the binding of elastin to cathepsins.

**DISCUSSION**

Besides matrix metalloproteinases (8, 11), cathepsins of the papain family have been recently implicated in atherogenesis and vascular remodeling. Focus has been given to the elastolytic activities of cathepsins S and K. Both cathepsins are potent elastin-degrading proteases surpassing the in vitro activities of prototype elastases such as pancreatic, leukocyte, and metalloelastases (MMP-12). The expression of cathepsins S and K has been described in smooth muscle cells and macrophages, with both cell types being critical in the pathophysiology of the arterial vascular system (3, 45). Elastin is an integral component of the intima and responsible for the elasticity of the blood vessels. Degradation of the elastin matrix leads to a destabilization of the vessel structure eventually causing aneurysms or blood vessel ruptures. Weiss and co-workers (15, 46) have recently suggested that thiol-dependent cathepsins and the matrix metalloproteinase, matrilysin, are the major elastolytic enzymes in macrophages. Moreover, cathepsin-mediated elastolytic activities in conjunction with the down-regulation of the endogenous cathepsin inhibitor, cystatin C, in
atherogenic plaques have been implicated in plaque formation and plaque rupture (47). The nature of the individual elastolytic cathepsins, however, is unclear. Based on in vitro elastase activities, cathepsins K, S, and L have been considered as candidates. Human cathepsin K displayed the highest activity against elastin at pH of 5.5 followed with ~60–90% and 10–20% of that activity by cathepsins S and L (depending whether Congo Red or [3H]elastin were used as substrates). However, the absence of an overall reduction of the elastolytic activity in macrophages obtained from a cathepsin K-deficient pycnodysostosis patient suggested that cathepsin K only insignificantly contributed to the degradation of [3H]elastin or that other cathepsins compensated for the lack of cathepsin K (15). It has been concluded that cathepsins S and probably L are the main elastolytic cysteine proteases in macrophages and that their activities correspond to ~60% of the total elastase activity.

In this report, we characterize cathepsin V as a novel and extremely potent elastase expressed in macrophages. Previous reports have restricted the expression of cathepsin V to the thymus, testis, and the cornea (16–18). Contrary to cathepsin S, which appears to be constitutively expressed in MDMs, cathepsin V is only detectable in activated macrophages. Significant cathepsin V expression was also observed within intimal plaque specimens. Here, we demonstrate that cathepsin V but not cathepsin L is the second major elastolytic activity in macrophages. Using inhibitors discriminating between cathepsins S, K, and other cathepsin L-like cysteine proteases, we could show that cathepsin V contributed as much as 20% of the total cysteine protease-dependent elastase activity of macrophages. Although the expression level of cathepsin V was only about 25% of that of cathepsin S in macrophages, its 4-fold higher elastase activity compensated for that. With a 3–4× higher [3H]elastin degrading activity than cathepsins K and S and a 2–6-times higher degradation rate than pancreatic elastase and MMP-12, cathepsin V represents by far the most potent elastase activity in mammalian cells yet described. Since cathepsin V as most other cathepsins with the exception of cathepsin S is labile at neutral pH (16), its main physiological activity might be intracellular. A predominant intracellular function is also supported by the failure to detect secreted cathepsin V. Neither the precursor nor the catalytically active forms could be identified in culture supernatants of macrophages by immunoblotting (data not shown). In contrast, cathepsins S and K expression has been detected in culture supernatants of macrophages by us (data not shown) and others (15). Punturieri et al. suggest that the degradation of insoluble elastin occurs extracellularly in an acidified microenvironment at the macrophage-elastin interface. They could demonstrate an acidification of the interface between the outer cell membrane and extracellular elastin particles thus identifying a microenvironment suitable for neutral pH labile cathepsins. Alternatively, it is also possible that elastin degradation, at least partially, occurs intracellularly after phagocytosing insoluble elastin particles. This mechanism is supported by our finding that the cell-permeable inhibitor, E-64d, which is inactive outside cells, inhibits ~20% of the total elastase activity of MDMs. This reduction in elastin degradation corresponds to the 20% contribution of cathepsin V. Since cathepsin V was not secreted by MDMs it can be speculated that the intracellular cathepsin-mediated elastase activity is mostly mediated by cathepsin V. The remaining 40% of the total elastase activity can thus be attributed to cathepsins S and K, which may equally contribute to the extracellular degradation of elastin based on the inhibitor studies described here. However, it should be noted that at very high concentrations of E-64 (100 μM) both, the intra- and extracellular elastase activities are inhibited. Thus, the attribution of about 60% of the elastase activities to extracellular cathepsin activities as previously suggested (15) might be an overestimation. As shown in this report, the addition of 100 μM E-64 led to an uptake of the inhibitor by cells resulting in the intracellular inhibition of cathepsins. It is tempting to speculate that in cathepsin K-deficient macrophages, cathepsin K activity is compensated by an increase of intracellular cathepsin V activity because the presence of 100 μM E-64 does not permit discrimination between extra- and intracellular activities. That primarily cathepsin V and not cathepsin S is up-regulated in pycnodysostosis can be argued based on our observation that the expression of cathepsin V but not that of cathepsin S is inducible in macrophages (Fig. 2, A and B).

The intracellular degradation of extracellular matrix components as a significant degradation pathway is rather underappreciated. Since macrophages are professional phagocytosing cells (48), it is plausible that they can uptake larger elastin fragments and degrade them intracellularly. The uptake of insoluble elastin, however, may require a specific extracellular elastase activity to liberate “phagocytosable” fragments from the highly cross-linked elastin. This could be achieved either by neutral pH stable elastases such as MMP-12, leukocyte elastase, and/or cathepsin S or in an acidified extracellular microenvironment by secreted cathepsins K and S. That macrophages can indeed uptake insoluble elastin particles was demonstrated by the detection of rhodamine-labeled elastin within the cells (Fig. 7, G and H).

Cysteine protease activity is regulated on the transcriptional/translational level and by various endogenous peptide inhibitors such as cystatins and certain serpins (49, 50). In addition, we have recently demonstrated that the degradation of extra-cellular matrix proteins by cathepsins is efficiently controlled by GAGs. GAGs are abundantly expressed in the extracellular matrix of blood vessel walls (51). The collagenase activity of cathepsin K requires the formation of specific complexes with chondroitin sulfate whereas other GAGs such as dermatan sulfate, heparan sulfate, and heparin strongly inhibit its collagenase activity (52). The collagenolytic activities of other cathepsins are completely suppressed by all other GAGs. In this report we demonstrated that also cathepsin V was capable to form a stable complex with chondroitin sulfate. The elastolytic activities of cathepsins K and V are strongly inhibited whereas the activity of the non-complex forming cathepsin S is not influenced. Complex formation with defined GAGs is necessary for the collagen degradation by cathepsin K but hampers the elastolytic activities of cathepsins K and V. In contrast to cathepsin S, cathepsins K and V are not constitutively expressed in monocytes and are induced only during maturation into macrophages. The inhibition of those two enzymes by GAGs may constitute an additional protective mechanism against excessive elastin degradation during inflammatory processes whereas cathepsin S may have a housekeeping function in elastin turnover. During atherogenesis, however, a depletion of the GAG content in blood vessel walls has been observed (53) which in turn may lead to an increase of the elastolytic activities of inducible cathepsins K and V and thus to an exaggeration of the blood vessel wall erosion.

<table>
<thead>
<tr>
<th>Condition</th>
<th>kcat</th>
<th>K_m</th>
<th>kcat/K_m</th>
</tr>
</thead>
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<tr>
<td>NaCl (0.3 M)</td>
<td>3.4 ± 0.2</td>
<td>4.8 ± 0.8</td>
<td>708,000</td>
</tr>
<tr>
<td>NaCl (0.15% w/v)</td>
<td>3.5 ± 0.2</td>
<td>4.9 ± 0.8</td>
<td>714,000</td>
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<tr>
<td>NaCl/NaCl</td>
<td>3.4 ± 0.2</td>
<td>4.8 ± 0.6</td>
<td>708,000</td>
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</tbody>
</table>
Elastolytic Activity of Cathepsin V

In summary, we have demonstrated that cathepsin V is expressed in MDMs and human atheroma tissues and that this protease displays the most potent elastolytic activity among mammalian proteases. It appears to be responsible for the bulk of the lysosomal elastin degradation and may contribute up to 20% of the total or 33% of cathepsin-mediated elastolytic activities in MDMs. Of particular interest is the finding that cathepsins V and K are efficiently inhibited by glycosaminoglycans. Reduction of GAG contents in atherosclerotic plaques may lead to a selective increase of both cathepsin activities and thus to an acceleration of the destruction of the elastin matrix in atherosclerotic arteries.

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REFERENCES