Expression of the Elastolytic Cathepsins S and K in Human Atheroma and Regulation of their Production in Smooth Muscle Cells

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Abstract

Formation of the atherosclerotic intima must involve altered metabolism of the elastin-rich arterial extracellular matrix. Proteases potentially involved in these processes remain unclear. This study examined the expression of the potent elastases cathepsins S and K in human atheroma. Normal arteries contained little or no cathepsin K or S. In contrast, macrophages in atheroma contained abundant immunoreactive cathepsins K and S. Intimal smooth muscle cells (SMC), especially cells appearing to traverse the internal elastic laminae, also contained these enzymes. Extracts of atheromatous tissues had approximately twofold greater elastase-specific activity than extracts of uninvolved arteries, mostly due to cysteine proteases. Cultured human SMC displayed no immunoreactive cathepsins K and S and exhibited little or no elastolytic activity when incubated with insoluble elastin. SMC stimulated with the atheroma-associated cytokines IL-1β or IFN-γ secreted active cathepsin S and degraded substantial insoluble elastin (15–20 μg/10^6 cells/24 h). A selective inhibitor of cathepsin S blocked > 80% of this elastolytic activity. The presence of cathepsins K and S at sites of vascular matrix remodeling and the ability of SMC and macrophages to use these enzymes to degrade elastin supports a role for elastolytic cathepsins in vessel wall remodeling and identifies novel therapeutic targets in regulating plaque stability. (*J. Clin. Invest.* 1998. 102:576–583.) Key words: atherosclerosis • cysteine protease • smooth muscle cell • elastolytic activity

Introduction

Remodeling of the extracellular matrix of blood vessels, and in particular, the degradation of elastin, contributes importantly to numerous aspects of formation and complication of atherosclerotic lesions. Early in formation of the thickened intima, smooth muscle cells (SMC)[1] that may migrate from the tunica media into the developing intima must penetrate the internal elastic lamina. The compensatory enlargement characteristic of the evolving atherosclerotic lesion must likewise involve remodeling of constituents of the arterial extracellular matrix, including elastin. Recent clinical and pathological evidence suggests that inflammatory cells, especially macrophages, accumulate in regions of plaques most susceptible to dissolution and rupture (1–3). The mechanisms of degradation of the arterial extracellular matrix in these various contexts remain unclear. Substantial evidence supports the involvement of matrix metalloproteinases (MMP) in this process (4–6). Some studies have also implicated serine proteases in vascular remodeling (7, 8). Cysteine proteases have received much less consideration in this regard, even though macrophages and SMC with greatly expanded lysosomal compartments figure prominently in the histopathology of atherosclerotic plaques.

Cysteine proteases have several properties that suggest that they might contribute to arterial remodeling during atherogenesis, if they were expressed in these lesions. Several cysteine endoproteases, i.e., cathepsins K, L, and S, have potent elastolytic activity (9–12). Cathepsin K is the most potent mammalian elastase yet described (13, 14), and also possesses collagenolytic activity (15). Although all members of this enzyme family have acidic pH optima, cathepsin S is stable at neutral pH, where it retains nearly as much elastase activity as leukocyte elastase (10, 11, 16, 17). Although these enzymes are thought to generally reside in and function optimally within acidic lysosomes, recent evidence indicates that these elastases may also function extracellularly at or near the cell surface. Monocyte-derived human macrophages become markedly elastolytic during in vitro culture and predominantly use cysteine proteases to degrade extracellular elastin (12). Acquisition of a degradative phenotype correlates with the ability of the macrophages to contact elastin, create an acidic pericellular space, and secrete elastolytic cysteine proteases, especially cathepsins S and L, into the contact zone (12, 18). This degradative mechanism is reminiscent of the bone macrophage, osteoclasts. Indeed, in osteoclasts, cathepsin K appears to be the major protease involved in extracellular matrix metabolism necessary for normal bone growth and remodeling (13). Deficiency of this enzyme results in accumulation of bone matrix mostly around rather than in osteoclasts, consistent with the primary site of action of cathepsin K in the pericellular space (19). Together, these studies establish that under certain conditions macrophages utilize the elastolytic cathepsins to re-

1. *Abbreviations used in this paper:* E64, *trans*-epoxysuccinyl-L-leucyl-amido-(4-guanidino)butane; LHVS, morpholinurea-leucine-homo-phenylalanine-vinylsulfone-phenyl; MMP, matrix metalloproteinase; SMC, smooth muscle cells.

model extracellular matrix, although cathepsin K protein has only been observed in bone, one study generally demonstrated cathepsin K mRNA and protein expression in human breast carcinoma (20).

Atherosclerotic plaques contain abundant macrophages. These cells accumulate at sites of weakening of the extracellular matrix, which predispose to plaque rupture. Disruption of atherosclerotic plaques frequently causes thrombosis and precipitates the acute manifestations of atherosclerosis, including unstable angina and acute myocardial infarction (2, 3). In view of the proteolytic potential of cathepsins K and S, and their possible presence in the very cell types found at sites of plaque liability, we investigated the expression of these proteases in normal and atherosclerotic human arteries and their regulation and function in vitro in cultured vascular SMC.

Methods

Human tissue. Atherosclerotic plaques from human coronary ($n = 9$) and carotid ($n = 15$) arteries and nonatherosclerotic arteries ($n = 12$) were obtained from transplantation donors, at endarterectomy, and at autopsy. Immunohistochemical localization of cathepsin S or K did not depend on whether frozen or paraffin sections were studied.

Immunocytochemistry. Serial cryostat sections after fixing in cold acetone at $-20°C$ for 5 min or paraffin sections after deparaffinization in xylene and rehydration in graded ethanols were stained using the avidin-biotin-peroxidase method. The reaction was visualized with 3-amino-9-ethyl carbazole as substrate (Sigma Chemical Co., St. Louis, MO). Staining for cathepsin S required 10 min pretreatment with 1 N HCl at room temperature before incubation with the primary antibodies. Sections were counterstained with Gill's hematoxylin solution.

Cell types were identified using monoclonal anti–muscle actin HHF-35 (Enzo Diagnostics, Syosset, NY); monoclonal anti–human macrophage HAM-56, and CD-68, monoclonal anti–human leukocyte common antigen (DAKO Corporation, Carpinteria, CA). Rabbit polyclonal antibodies against human cathepsins S and K were produced as described previously (21, 22), and affinity purified using immobilized antigen. To control for antibody specificity, immunohistochemical studies were performed in parallel with a preparation of antibody that had been incubated overnight at 4°C with a 20-fold excess of antigen.

Cell culture. SMC obtained from human saphenous veins were subcultured at passages 2–5 into four- or eight-chamber slides (Lab-Tek, Naperville, IL) for immunostaining or into 150-cm² flasks (Corning, Inc., Corning, NY). SMC were grown to confluency in DME (Bio Whittaker, Walkersville, MD) containing 100 U/ml penicillin B, 100 μg/ml streptomycin, and 1.25 μg/ml amphotericin and supplemented with 10% FCS. Cells were stimulated for 24 h with recombinant IL-1β (10 ng/ml; Genzyme Corp., Cambridge, MA), recombinant human TNF-α (10 ng/ml; Genzyme Corp.), or human recombinant IFN-γ (400 U/ml; Becton Dickinson Labware, Bedford, MA). Stock solutions of these cytokines contained 0.1% pyrogen-free human serum albumin as a carrier.

Western blot analysis. Frozen tissues from four nonatherosclerotic arteries and five carotid plaques were pulverized and lysed (0.3 μg tissue per milliliter of lysis buffer) as described below. After 1 h incubation at 37°C, the lysates were clarified by centrifuging at 16,000 *g* for 15 min. The protein concentration for each lysate was determined using a protein assay system according to the manufacturer (Bio-Rad Dc; Bio-Rad Laboratories, Hercules, CA). 300 μg of protein from each sample were separated on 12% SDS-PAGE and blotted onto nitrocellulose filter (Schleicher and Schuell, Keene, NH). The filter was then incubated with 5% nonfat milk/PBS overnight to block nonspecific binding sites. Immunoblotting used immunopurified antibodies against human cathepsins S or K (1:200 dilution) (19, 21). Blots were developed using chemiluminescence (ECL system; New England Nuclear, Boston, MA).

Northern blot analyses. Four nonatherosclerotic aortas and five atherosclerotic carotid plaques were pulverized and total RNAs precipitated from a modified guanidium isothiocyanate lysate with isopropanol according to the manufacturer's instructions (Stratagene Inc., La Jolla, CA). Total RNAs were dissolved in diethylpyrocarbonate-treated distilled water and 20 μg of total RNA from each sample was separated on 1.2% agarose gels followed by electroblotting onto membrane (Maximum Strength Nytran Plus; Schleicher and Schuell). To analyze SMC RNA, 15,000,000 SMCs from each culture were lysed in guanidinium isothiocyanate lysis buffer and the RNA was prepared as described previously (22, 23). RNA blots were then prehybridized and hybridized with α-[³²P]dCTP (New England Nuclear) labeled cathepsins S and K full-length cDNA fragments, and glyceraldehyde-3-phosphate dehydrogenase cDNA fragment (Clontech, Palo Alto, CA) respectively, in Express Hyb Solution (Clontech) for 1 h followed by sequential 20 min of washing in 2× SSC/2% SDS, 0.5× SSC/0.5% SDS, and 0.1× SSC/0.1% SDS, respectively. Membranes were then exposed to X-omat film (Eastman Kodak, Rochester, NY), and the autoradiograms were quantified by densitometric analysis using National Institutes of Health Image software.

Elastase assays. Tissue from five nonatherosclerotic aortas and seven carotid plaques was snap-frozen in liquid nitrogen and stored at $-80°C$ until study. Tissue samples were pulverized in a liquid nitrogen-cooled aluminum mortar and divided into two portions. One half, ~ 0.2 g of tissue, was lysed in 0.5 ml of buffer optimized for acidic protease activity of cathepsins (11) containing 1% Triton X-100, 40 mM sodium acetate, and 1 mM EDTA, pH 5.5; and another half was lysed in a buffer optimized for a study of neutral MMP activity (5), 10 mM phosphate, pH 7.2, containing 150 mM Na chloride (PBS), and 1% Triton X-100, 0.015 M CaCl₂, 0.1% SDS, 0.5% Na deoxycholate, and 0.2% Na azide (PBSTS). SDS was included during assay to optimize elastolytic activity (24). The tissue was extracted at 37°C for 1 h, and the lysates were then collected by centrifuging at 16,000 *g* for 10 min. Tissue lysate elastolytic activity was assayed as described previously (11). Briefly, 50 μg total protein in 10 μl of 150 mM DTT, and 240 μl of lysate assay buffer containing 0.05% Triton X-100, 20 mM sodium acetate, and 1 mM EDTA, pH 5.5, were added onto the 24-well plate coated with 200 μg of [³H]elastin per well. The same amount of total protein in PBSTS buffer, pH 7.2. All assays were performed in duplicate. [³H]Elastin (bovine ligamentum nuchae; Elastin Products, St. Louis, MO) was prepared as previously described (24). The specific activity of the preparation was 700 cpm/μg. The plate was incubated overnight at 37°C. Samples were then collected and spun at 16,000 *g* for 15 min to separate soluble product from undegraded, insoluble elastin. The soluble radioactivity was measured by liquid-scintillation spectroscopy. Parallel incubations with inhibitors were used to distinguish the contribution to elastin degradation of cysteine proteases (20 μM E64 [*trans*-epoxysuccinyl-L-leucylamido-{4-guanidino} butane]) or MMPs (20 mM EDTA), respectively. All reagents were purchased from Sigma Chemical Co., unless stated otherwise.

SMC were assayed for elastase activity in cocultures with insoluble [³H]elastin. SMCs obtained from saphenous veins were seeded on 24-well plate (Becton Dickinson, Lincoln Park, NJ) at 1×10^5 cells per well in 1 ml of DME/10% FCS. After stimulating the cells with 500 U/ml IFN-γ or 10 ng/ml IL-1β for 24 h, 200 μg of [³H]elastin (1300 cpm/μg) was added into each well in the absence and presence of protease inhibitors including E64d (20 μM; Sigma Chemical Co.), LHVS (morpholinurea-leucine-homophenylalanine-vinylsulfone-phenyl, 5 nM; Arris Pharmaceutical, Inc., South San Francisco, CA), TIMP-1 (100 ng/ml; Oncogene, Cambridge, MA), and PMSF (1 mM; Sigma Chemical Co.). Cells without IFN-γ stimulation were treated in the same way as stimulated cells. After further incubation at 37°C for 4 d, media were collected and centrifuged at 16,000 *g* for 15 minutes. Supernatant (200 μl) was carefully transferred into 5 ml of aquasol-2 for scintillation counting of digested [³H]elastin. Each treatment was performed in four separate wells and each well was counted in

duplicate. Results were presented as mean±SD of three separate experiments.

Elaboration of active cathepsin S by cultured SMC. Human saphenous vein SMC were cultured in serum-free medium (25) for one day before stimulation with IFN-γ as described above. Stimulation was carried on continuously for 4 d in the presence of [^{125}I]tyrosine-alanine-diazomethylketone ([^{125}I]tyr-ala-CHN$_2$), which irreversibly binds and inhibits active cysteine proteases (12). This probe should label both intracellular and extracellular active cysteine proteases. The culture medium was then collected and concentrated by precipitation with TCA (5%) in the presence of 0.3% bovine calf serum. Samples were neutralized and both the protein pellets and cells solubilized in Laemmli sample buffer and immunoblotted for cathepsin S as described above. The blot was then exposed to X-Omat film (Eastman Kodak) to visualize [^{125}I]tyr-ala-CHN$_2$-labeled cysteine proteases. As control, released lactate dehydrogenase activity in the medium was monitored spectrophotometrically according to the manufacturer's instructions (Sigma Diagnostics). Immunoblots were also reprobed with antibodies to an additional cytoplasmic enzyme marker, mitogen-activated kinase (New England Biolabs, Boston, MA) to assess nonspecific release of intracellular contents during the cultures.

Results

Foam cells in human atheroma contain immunodetectable cathepsins S and K. In nonatherosclerotic human arteries ($n = 12$), immunohistochemical evaluation showed negligible expression of cathepsins S or K protein (Fig. 1, *top*). In atheromatous carotid or coronary arteries ($n = 24$), we observed different patterns of expression of these cathepsins depending on the morphology of the particular lesion in less fibrous, nonocclusive lesions ($n = 7$) immunoreactive cathepsins S and K tended to localize not only in regions of the expanding intima but also in the subjacent medial smooth muscle cells (Fig. 1, *middle*). In contrast, medial SMC in the normal arteries ($n = 9$) consistently lacked cathepsin S and K expression (compare Fig. 1, *top* to Fig. 1, *middle*). In advanced fibrous plaques ($n = 17$) (Fig. 1, *bottom*), cathepsins K and S localized in the intima both in the fibrous cap and in regions of foam cell accumulation, in the shoulders of lesions more prominently than in the lipid-rich core. Interestingly, in the more advanced plaques, the underlying medial SMC appeared to express less cathepsin S than they did in less fibrous, raised lesions, which likely represent an earlier stage in lesion evolution. Both snap-frozen and paraffin-embedded tissue yielded similar findings.

We found cathepsin K-positive SMC-derived foam cells in the fibrous cap of atheromata (Fig. 2, *top left*), and in the macrophage-derived foam cells in the shoulder area of these lesions (Fig. 2, *bottom left* and *right*). The lack of reaction with an antibody pre-absorbed with an excess of recombinant cathepsin K established the specificity of staining under the conditions used in these experiments (Fig. 2, *top right*). In all atherosclerotic plaques examined ($n = 24$), macrophages always contained immunodetectable cathepsins S and K regardless of the plaque morphology, or the abundance of macrophages. Two-color immunohistochemical analysis substantiated the localization of cathepsin S and cathepsin K in both smooth muscle cells and macrophages (data not shown).

At the earlier stages of atherogenesis, such as diffuse intimal thickening ($n = 3$) and fatty streaks ($n = 4$), we frequently observed areas with interruption and fragmentation of the internal elastic lamina (Fig. 3, *middle*). Interestingly, medial SMC appearing to migrate into the intima contained both
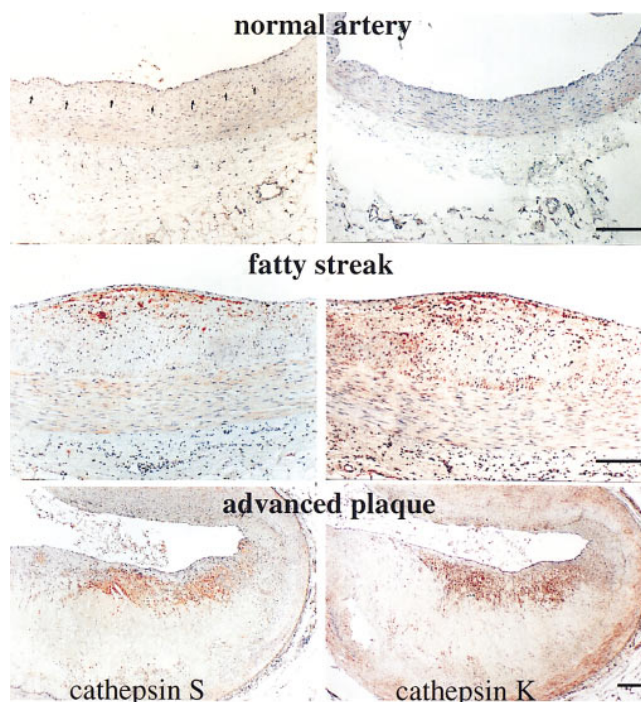

Figure 1. Cathepsin S (*left*) and cathepsin K (*right*) distribution in normal and atheromatous arteries, disclosed by the red–brown staining due to the aminoethyl carbazole reaction product. A nonatherosclerotic artery (*top*) contains few if any cathepsin S- or K-positive cells. Fatty streaks (*middle*) have cathepsin S- and K-positive cells in the expanded intima as well as in medial SMC. In more advanced fibrous plaques (*bottom*), cathepsin-containing cells localized in the fibrous cap, the SMC of the tunica media show weak or no staining. These results are representative of findings in 12 nonatherosclerotic arteries and 24 atherosclerotic lesions studied. *Arrows*, internal elastic lamina. (*Bars*, 200 µm).

cathepsins tested (illustrated here by staining for cathepsin K, Fig. 3, *bottom*).

Expression of cathepsins S and K protein and mRNA in atheroma vs. normal arterial tissue. Immunoreactive cathepsin K protein colocalizes with cathepsin S within atheroma (Figs. 1–3). Extracts of atheroma ($n = 5$) and normal arteries ($n = 4$) were analyzed by Western and Northern blotting. Immunoblotting (Fig. 4 *A*) confirmed increased cathepsin S and K proteins in atheroma. The apparent sizes of the proteins were 28 and 29 kD, respectively, consistent with the reported sizes of the active forms of these cathepsins (11, 22). Densitometric analysis of Northern blotting (Fig. 4 *B*) showed a three- to fourfold increase in cathepsin S mRNA in extracts of atheroma. Interestingly, similar levels of cathepsin K mRNA were found in both atherosclerotic and normal tissues.

Atheroma extracts have elevated elastase activity. To test whether the cathepsins S and K expressed by foam cells in atheroma can actually degrade an extracellular matrix component, we tested the elastolytic activity of tissue extracts from nonatherosclerotic arteries ($n = 5$) and atherosclerotic plaques ($n = 7$). When assayed under conditions that optimize cysteine protease activity, plaque extracts showed almost twofold higher acidic, SH-dependent elastolytic activity than those
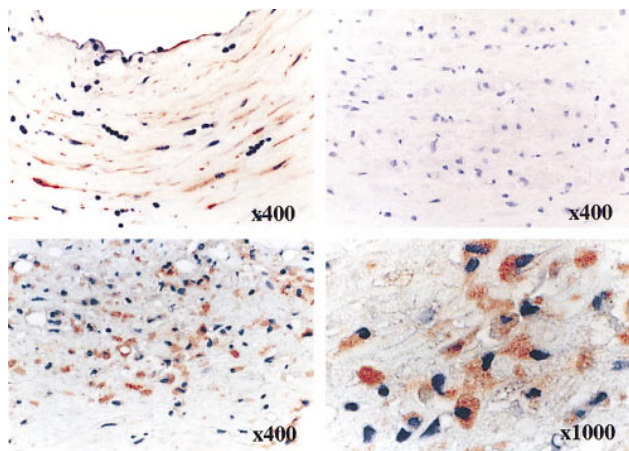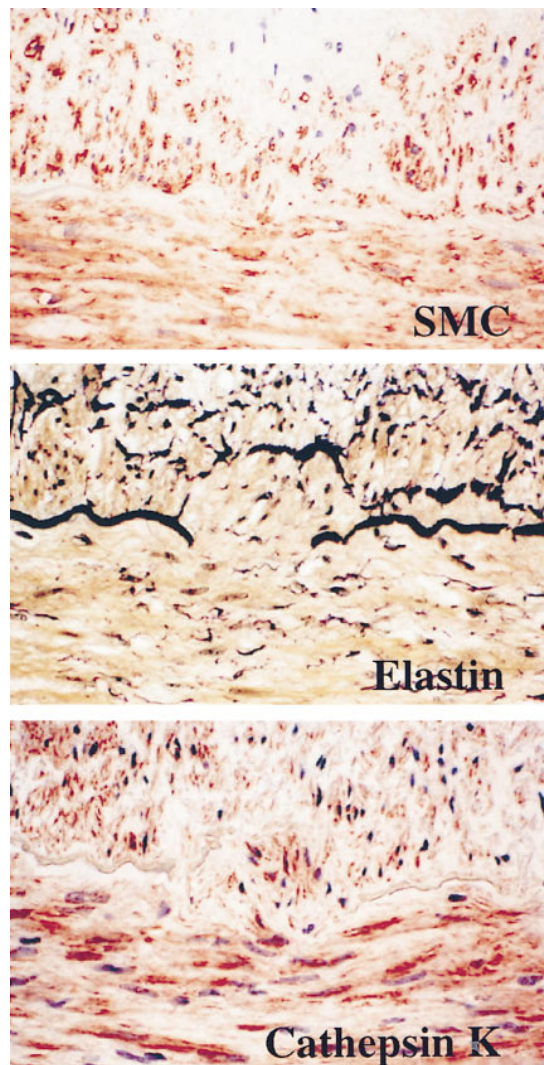
Figure 2. Cathepsin K expression in human atheroma (generated by using an affinity-purified rabbit anti–human cathepsin K antibody). SMC in the fibrous cap (*top left*) contain cathepsin K protein. In all atherosclerotic plaques examined, macrophages in the shoulder area contain immunodetectable cathepsin K (*bottom*). The lack of reaction with an antibody pre-absorbed with an excess of recombinant cathepsin K established the specificity of antibodies used under these conditions (*top right*).

Figure 3. Colocalization of cathepsin K-positive SMC with areas of internal elastic lamina fragmentation and medial SMC migration. Medial SMC and those that appear to migrate into the intima in fatty streaks (*top*, staining for α-actin, $n = 4$) colocalize with fragmentation of the internal elastic lamina (*middle*, Verhoff–van Gieson staining), and express cathepsin K (*bottom*) ×400.

from nonatheromatous arterial tissues (Fig. 5). Approximately 40% of the elastolytic activity from tissue extracts of atherosclerotic plaques was inhibited by the cysteine protease inhibitor E64. In contrast, when assayed under conditions that optimize MMP activity, neutral, metal-dependent elastolysis of the radioactive substrate was much lower and showed no significant difference for extracts from atherosclerotic vs. uninvolved tissue. Approximately 50% of the activity in the lysates was due to neither cysteine proteases nor metalloproteases, and this activity did not differ between atherosclerotic and normal tissues (Fig. 5). The basis for this activity remains undefined.

SMC express cathepsins K and S protein and mRNA. Previous studies have established the capacity of macrophage/osteoclasts to express cathepsins S and K (11, 14, 19). The in situ findings reported above suggested that SMC might also synthesize these enzymes within human atheroma. Alternatively, the immunoreactive cathepsins associated with arterial SMC in the diseased arteries may have bound to smooth muscle cells after elaboration by neighboring macrophages, a cell type known to express these enzymes. We therefore tested the capacity of cultured SMC, free of mononuclear phagocytes, to express these cathepsins.

Under usual culture conditions, human vascular SMC display no immunoreactive cathepsin S (Fig. 6, *top*). However, after exposure to IL-1β (10 ng/ml, 24 h) the cultured SMC uniformly contain immunoreactive cathepsin S (Fig. 6, *middle*). We obtained similar results with TNF-α (10 ng/ml, 24-h exposure) and IFN-γ (400 U/ml, 24 h) (data not shown). The IL-1β-stimulated SMC cultures showed no staining with anti–cathepsin S antibodies, which were preabsorbed with recombinant protein (Fig. 6, *bottom*). In contrast, we found very low and similar levels of immunoreactive cathepsin K expression in VSMC with or without IL-1β stimulation (data not shown). Thus, under these culture conditions, cytokine-stimulated SMC predominantly express cathepsin S.
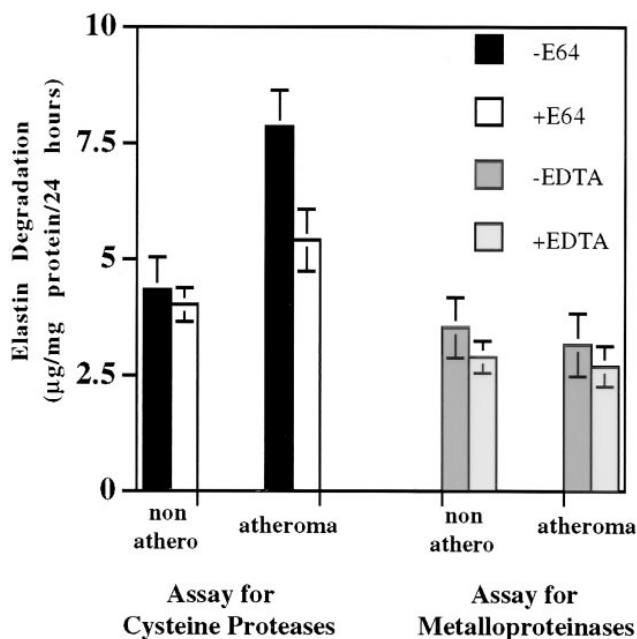
To characterize more completely the ability of cytokines implicated in atherogenesis to regulate the cathepsin S gene expression in SMC, we performed Northern blot analyses of total RNA from human vascular SMC cultured under the same conditions. We found that treatment with IFN-γ (400 U/ml, 24 h), and IL-1β (10 ng/ml, 24 h) increased the expression of cathepsin S mRNA > 6-, and 3.3-fold, respectively, as determined by densitometric analysis, while unstimulated SMC expressed very low levels of this mRNA (Fig. 7). Cathepsin K mRNA showed similar levels of expression under basal conditions, and after cytokine stimulation (Fig. 7). In the context of cytokines as a potential stimulus for cathepsin S mRNA and protein expression, we colocalized immunoreactive IL-1β and cathepsin S in areas rich in macrophage-derived foam cells within the shoulder region of atherosclerotic plaques (data not shown).
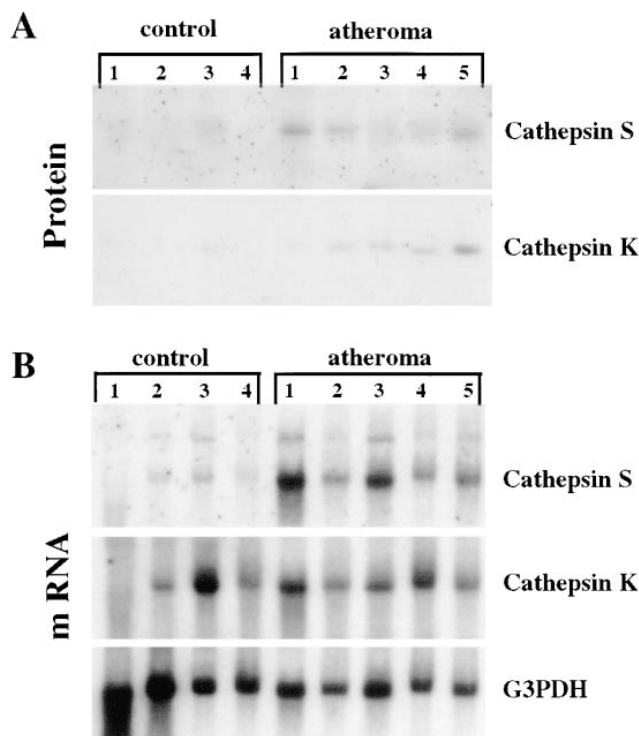
Figure 4. (*A*) Western blot analysis of protein extracts from both human normal and atherosclerotic arteries. An equal amount of protein for each sample was loaded and immunoblotted using rabbit antibodies against human cathepsins S and K (1:200 dilution). Both a 28-kD cathepsin S band and a 29-kD cathepsin K band, consistent with the sizes of the active enzymes, were visualized. (*B*) Northern blot analysis of human cathepsins S and K in normal arteries and atheromas. 20 μg of total RNA were used for each sample and the blot was probed sequentially with labeled cathepsin S and K cDNAs and then with glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) cDNA as a control for RNA loading.


Figure 5. Effect of selective inhibitors on cathepsin and metal-dependent elastolytic activity of tissue extracts from human atheroma vs. nonatherosclerotic arteries (*non-athero*). Elastase activity was determined as described in Methods. Tissue extracts without inhibitors (−) or mixed with inhibitors (+) were incubated with ^{3}H-labeled elastin for 24 h at 37°C. The soluble radioactivity was measured by liquid scintillation spectroscopy as counts per minute per microgram protein. The selective inhibitors E64 (20 μM), or EDTA (20 mM) were used to determine degree of elastin degradation by cysteine proteases, and MMPs, respectively.

IFN-γ–stimulated vascular SMC degrade elastin. To test the function of cysteine elastases in SMC, we assayed the ability of the intact cells to degrade elastin. Cultured SMC exhibit little or no elastolytic activity under basal conditions (Fig. 8 *A*). Exposure to IFN-γ (400 U/ml for 24 h) substantially augmented elastin degradation by SMC cultures over the ensuing 4 d. The comparison of Figs. 7 and 8 shows a direct correlation between the induction of cathepsin S expression and elastolytic activity of cultured SMC. Further, a selective cathepsin S and K inhibitor, LHVS, as well as an inhibitor of all papain-like proteases, E64, reduced elastolytic activity > 80%. TIMP-1 and PMSF reduced elastin degradation to a much lesser extent (Fig. 8 *A*). Cultured SMC stimulated by IL-1β (10 ng/ml) also showed elevated elastolytic activity as compared with nonstimulated control (8.7±0.2 vs. 3.9±0.5 μg of elastin/10^6 cells/24 h). About 75% of this activity was inhibited by E64, and < 30% was inhibited by TIMP-1 or PMSF.

Elastin degradation by intact SMC could be due to either extracellular release of active cathepsin S or internalization of small amounts of elastin followed by intracellular degradation.

To determine whether cytokine-stimulated SMC secrete an active form of cathepsin S, supernatants of control and cytokine-stimulated cells were concentrated and analyzed for cathepsin S by immunoblotting. No detectable cathepsin S was released by unstimulated SMC, whereas the 4-d supernatant of IFN-γ–stimulated SMC did contain the 28-kD active form of cathepsin S (Fig. 8 *B*). That the released cathepsin S was active was verified by its binding [^{125}I]tyr-ala-CHN$_2$, an active-site inhibitor of papain-type cysteine proteases (Fig. 8 *B*). Because this inhibitor penetrates cells and could be incorporated into cathepsin S intracellularly, we cannot be sure whether cathepsin S is secreted in an active form or activated from its proform after release. Notably, lysates of SMC contained more cathepsin B than S, as judged by active-site labeling, whereas supernatants contained much more cathepsin S (Fig. 8 *B*), suggesting secretion rather than cell death as a source of released enzyme. This interpretation was supported by assays of released lactate dehydrogenase, which showed no increase during the 4 d of culture, as well as by the absence of immunoreactive mitogen-activated kinase, a cytoplasmic protein expressed in SMC, in the culture supernatants (data not shown). Thus, while we cannot exclude the possibility that elastin degradation by cytokine-stimulated SMC is in part due to internalization of elastin, our data show that stimulated SMC capable of mediating considerable elastin degradation can secrete active cathepsin S.
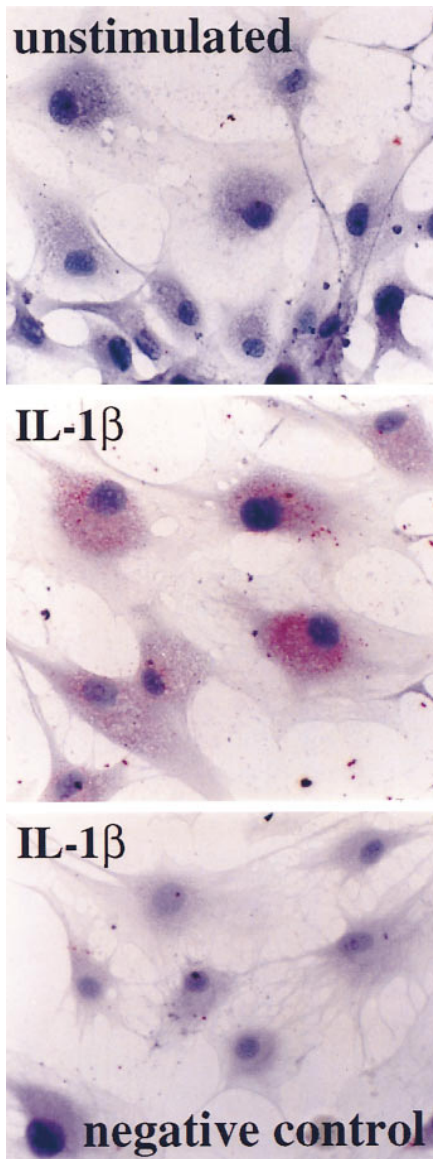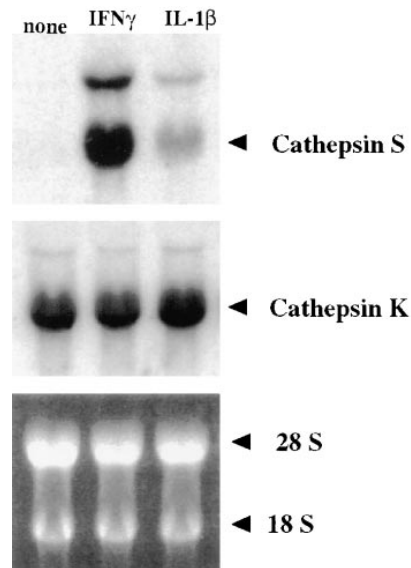
Figure 6. Cytokines stimulate cathepsin S production in cultured SMC. Unstimulated SMC (*top*) contain no immunoreactive cathepsin S, whereas those stimulated for 24 h with IL-1β (10 ng/ml, *middle*) show substantial staining for this enzyme. The bottom panel shows a control with pre-immune serum. ×400.

Figure 7. Northern blots of total RNA from vascular SMC cultured under different conditions. 30 μg of total RNA harvested from cells treated as noted were separated on 1.2% agarose gel and blotted onto a nylon filter. The filter was hybridized with both α-[^{32}P]dCTP-labeled cDNA probes for cathepsin S and cathepsin K, and exposed to X-Omat film (Eastman Kodak) at −80°C.

Discussion

Recent clinical and pathological findings have identified rupture of the fibrous cap of human atherosclerotic plaques as a major mechanism underlying coronary artery thrombosis (1, 25). Thus, the metabolism of the extracellular matrix components collagen and elastin, which confer structural integrity upon the lesion's fibrous cap, can critically influence the clinical manifestations of atherosclerosis. We and others have therefore studied regulation of the integrity of the arterial extracellular matrix. Abundant evidence now supports a potential role for MMPs in this context (4, 5, 26, 27).

This report demonstrates by immunostaining that cells that migrate into and accumulate within developing intimal plaques also synthesize the potent elastases, cathepsins K and S. These studies do not demonstrate secretion of active cathepsins K and S in situ, as there is no available signature for activities of these enzymes in vivo at present. However, we have verified that cytokine-stimulated vascular SMC can use cathepsin S to degrade extracellular, insoluble elastin in vitro (Fig. 8). Expression of this SMC elastolytic potential required activation by certain cytokines such as IFN-γ and IL-1β, found in developing plaques (28–30). In cultured rabbit articular chondrocytes (31), IL-1β increased production but not secretion of cathepsin B, and both IL-1β and TNF-α stimulated the secretion of a latent cysteine proteinase activity from human synovial fibroblast-like cells (32). Cells within atheroma frequently display markers of exposure to IFN-γ such as MHC class II molecules (33), consistent with our observation of expression of cathepsin S by lesional cells and our hypothesis that SMCs in developing plaques can utilize these enzymes in matrix remodeling.

In contrast with prior studies of elastase activities of vascular SMC, which utilized cell lysates (7, 8), we have examined the elastolytic potential of living SMC. Unstimulated SMC displayed little or no elastolytic activity but, in response to cytokine stimulation, degraded particulate elastin in a manner similar to that of human monocyte-derived macrophages, which also largely utilized cathepsins S and L for elastolysis (12). At least in vitro, SMC elastolytic activity was ∼ 10-fold less than that of stimulated macrophages (12). The capacity of stimulated SMC, like macrophages, to use these enzymes in extracellular matrix remodeling resembles the physiological behavior of osteoclasts (13, 34). Previous reports of accumulation of the bone matrix protein osteopontin in atheroma, and osteopontin expression by SMC after vascular wall injury, agree with concept of "activation" of SMC at sites of vessel wall remodeling and atherogenesis (35). Although the SMC used in

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B
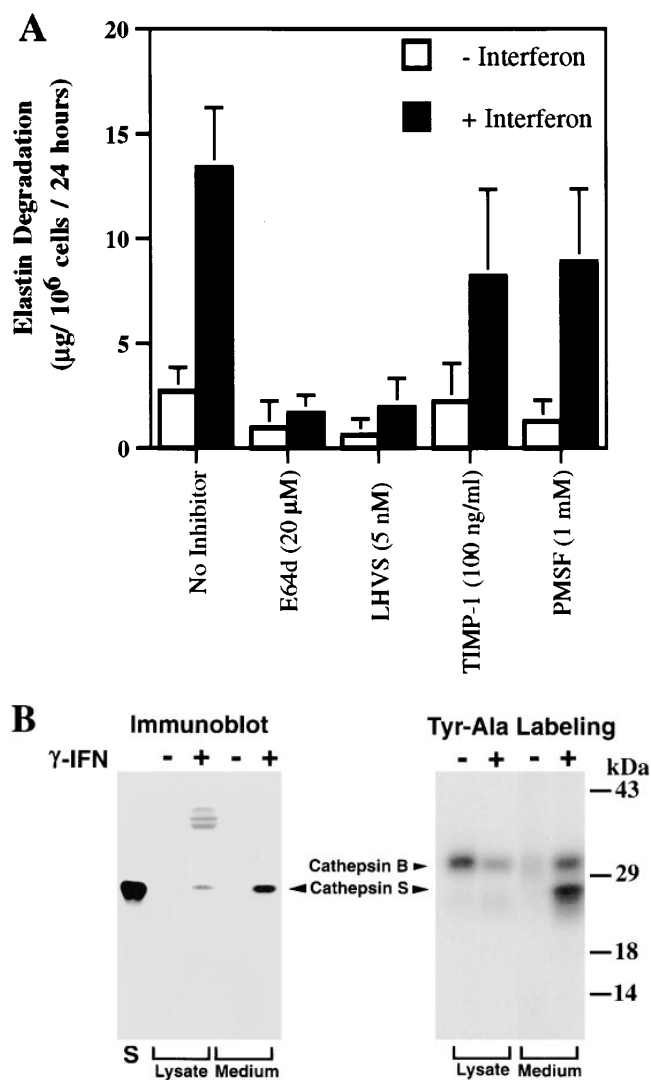
Figure 8. SMC secrete an active cysteine protease that can degrade extracellular elastin. (*A*) Elastin degradation by cultured human vascular SMC in the presence (*black bars*) or absence (*white bars*) of IFN-γ and various protease inhibitors as indicated in the figure. The relative amounts of elastin degradation are presented as micrograms of elastin degraded (mean±SD) per million cells in 24 h (mean±SD) from four independent experiments. (*B*) IFN-γ stimulates cultured SMC to express and secrete active cathepsin S. Cell lysates and protein precipitated from culture media were electrophoresed and immunoblotted for cathepsin S (*left*). Recombinant cathepsin S (*S*) marks the migration position of active enzyme. The higher molecular weight species present in the lysates of IFN-γ-stimulated cells are consistent with the known precursor forms of cathepsin S (11). Active cysteine proteases were present in both cell lysates and conditioned media as judged by active site labeling with [^{125}I]tyr-ala-CHN$_2$ (*right*).

our in vitro studies appear to express and secrete predominantly cathepsin S to degrade particulate elastin (Fig. 8), our immunostaining showed substantial immunoreactivity of cathepsin K in both SMC and macrophages within atheroma (Figs. 1 and 2). In the complex environment of the atheroma cathepsin K and potentially other elastolytic cathepsins such as cathepsin L (12) may contribute along with cathepsin S to vascular wall remodeling.

The increase in elastolytic activity observed in atheromata,

as compared with normal tissue (Fig. 4), was almost entirely due to papain-type cysteine proteases. This observation correlates well with our findings that atheroma contain large clusters of cathepsin K- and S-positive macrophages and SMC. Immunostaining and Western blot analyses disclosed little or no expression of these enzymes in the uninvolved arterial tissue. It is noteworthy that neither cathepsins nor metalloproteinases appear to account for all of the measured elastase activities. This residual activity, although not different between normal and diseased arteries, remains unexplained. Some or all of this activity could be due to serine proteases, which have previously been isolated from similar extracts of vessel walls and shown to contribute to elastase(s) in such extracts (7, 8).

The localization of cathepsin K in SMC and macrophages within atheroma establishes a novel source of this enzyme expression outside of bone. Analysis of the promoter region of cathepsin K reveals hallmarks of a weakly regulated gene at the transcriptional level, a conclusion supported by prior work demonstrating low constitutive levels of cathepsin K mRNA in numerous tissues (34, 36). However, cathepsin K protein was not observed in numerous normal tissues examined and it has been considered to be selectively expressed in bone. In contrast, as judged both by immunostaining and immunoblotting of whole tissue extracts, our data show the presence of cathepsin K protein within human atheroma. This finding implies that regulation of cathepsin K expression is likely posttranscriptional. The lack of cathepsin K protein in unstimulated cultured SMC, in spite of clear presence of its mRNA, supports this view.

Unlike cathepsin K, the levels of both cathepsin S mRNA and protein strongly increased in primary cultures of vascular SMC exposed to either IFN-γ or IL-1β. This response to cytokines is consistent with the highly regulated nature of the cathepsin S promoter (16, 21), and the response to IFN-γ consistent with prior proposals that cathepsin S may contribute to processing of antigen for MHC class II molecules (33). The response to IL-1β in vitro has potential in vivo relevance as immunoreactive cathepsin S and IL-1β colocalize in macrophage-derived foam cells within atheroma (data not shown).

The findings that atheroma contain abundant elastolytic cathepsins have potential therapeutic interest. These enzymes localize to sites of plaques prone to rupture and these cathepsins can mediate extensive matrix breakdown. The present observations suggest that even effective inhibition of MMP may not suffice to arrest enzymatic breakdown of the arterial extracellular matrix, and identify elastolytic cathepsins as an additional potential therapeutic target in this setting.

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