Lysosomal Cysteine Proteases in Atherosclerosis

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Abstract—Atherosclerosis is an inflammatory disease characterized by extensive remodeling of the extracellular matrix architecture of the arterial wall. Although matrix metalloproteinases and serine proteases participate in these pathologic events, recent data from atherosclerotic patients and animals suggest the participation of lysosomal cysteine proteases in atherogenesis. Atherosclerotic lesions in humans overexpress the elastolytic and collagenolytic cathepsins S, K, and L but show relatively reduced expression of cystatin C, their endogenous inhibitor, suggesting a shift in the balance between cysteine proteases and their inhibitor that favors remodeling of the vascular wall. Extracts of human atheromatous tissue show greater elastolytic activity in vitro than do those from healthy donors. The cysteinyl protease inhibitor E64d limits this increased elastolysis, indicating involvement of cysteine proteases in elastin degradation during atherogenesis. Furthermore, inflammatory cytokines augment expression and secretion of active cysteine proteases from cultured monocyte-derived macrophages, vascular smooth muscle cells, and endothelial cells and increase degradation of extracellular elastin and collagen. Cathepsin S–deficient cells or those treated with E64d show significantly impaired elastolytic or collagenolytic activity. Additionally, recent in vivo studies of atherosclerosis-prone, LDL receptor–null mice lacking cathepsin S show participation of this enzyme in the initial infiltration of leukocytes, medial elastic lamina degradation, endothelial cell invasion, and neovascularization, illustrating an important role for cysteine proteases in arterial remodeling and atherogenesis. **(*Arterioscler Thromb Vasc Biol*. 2004;24:1359-1366.)**

Key Words: atherosclerosis ■ cysteine proteases ■ cathepsins ■ cystatin ■ cytokines

The extracellular matrix (ECM) of the vascular wall, largely elastin and collagen, subserves many functions essential for vessel homeostasis. These macromolecules serve as an adhesive substrate for vascular endothelial cells (ECs) and smooth muscle cells (SMCs), furnish survival signals to resident cells, bind and retain lipoproteins, and provide a reservoir for growth factors.[1] Elastin and collagen also contribute to the strength, resiliency, and structural integrity of the vascular wall.[1,2] Normal tissues exhibit strict regulation of the expression and turnover of ECM. However, ECM damage or remodeling in conditions such as rheumatoid arthritis,[3] malignant tumors,[4] aortic aneurysm,[5,6] and atherosclerosis[7,8] contributes to the formation, progression, and clinical expressions of these disorders.

Atherosclerosis is an inflammatory disease whose complex developments encompass 3 main stages: initiation, progression, and complication.[8] Throughout these stages, several pathologic events involve proteolysis, including initial translocations of mononuclear leukocytes from the vascular lumen through the basement membrane into the subendothelium; progressive migration of SMCs through the elastic laminas from the media into the intima; and finally, disruption of the arterial wall—outward in aneurysmal disease and luminally in athero-occlusive disease.

Many studies have documented augmented elastase, collagenase, and gelatinase activities within atherosclerotic lesions. Among the responsible proteolytic enzymes, matrix metalloproteinases (MMPs) and serine proteases have garnered the most attention.[9–15] Increased expression of MMP-1, -2, -3, -7, -8, -9, and -13 and of metalloelastase-12 (MMP-12) occurs in macrophages bordering the lipid core adjacent to the fibrous cap and in macrophages and SMCs in the shoulder regions of well-developed atherosclerotic plaques, sites prone to rupture.[7,12,13,16] Studies in animals have permitted assessment of the contribution of these proteases to arterial remodeling. Administration of small-molecule MMP inhibitors delayed SMC migration and intima formation in injured rat arteries.[17] Overexpression of tissue inhibitors of MMPs (TIMPs) reduced atheroma progression[18] or intimal thickening in veins.[19] However, introduction of an MMP-1 transgene driven by a macrophage promoter into atherosclerosis-prone, apolipoprotein E (Apo E)–deficient mice surprisingly reduced atherosclerotic lesions[20] for reasons that remain uncertain. Human atherosclerotic lesions overexpress both urokinase- and tissue-type plasminogen activators.[21,22] Deficiency of urokinase-type plasminogen activator protected against medial elastic destruction and atherosclerotic aneurysm formation in Apo E–null mice, probably via impaired

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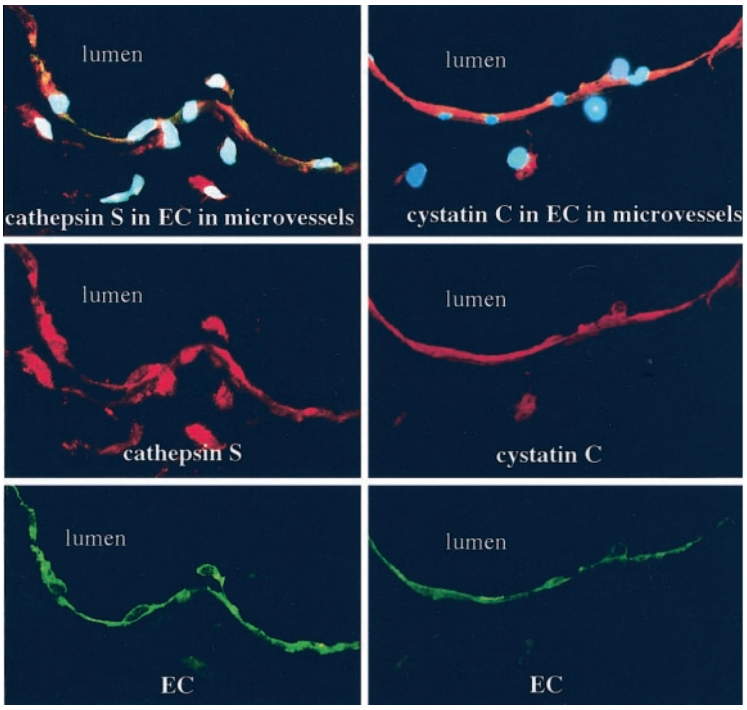

Figure 1. Colocalization of cathepsin S and cystatin C with microvascular endothelium. Frozen sections of human carotid lesions were double immunostained with rabbit anti-human cathepsin S (1:300)[33] or cystatin C (1:1500, Vortex) polyclonal antibodies followed by biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, Calif). After application of the avidin/biotin blocking kit, anti-CD31 monoclonal antibody (1:30, Dako) for ECs was added. Subsequently, biotinylated horse anti-mouse secondary antibody was applied, followed by streptavidin-fluorescein isothiocyanate (FITC, Amersham). Nuclei were counterstained with 0.5 μg/mL bis-benzimide H33258 (Calbiochem) in PBS. Both cathepsin S (red, left; $\times$400) and cystatin C (red, right; $\times$400) colocalized with endothelial marker CD31 (green). Orange color on the top panels indicates microvascular ECs expressing both antigens. Lumen of the microvessel is indicated.

plasmin-mediated elastolytic MMP activation.[23] In contrast, transgenic expression of rabbit urokinase-type plasminogen activator enlarged neointimal lesions and caused degradation of elastic laminas in cholesterol-fed rabbits.[24] However, plasminogen deficiency accentuated atherogenesis in atherosclerosis-prone, Apo E–null mice.[25] Recent findings have described expression of neutrophil elastase, a serine proteinase, in the macrophage-rich shoulders of human atherosclerotic lesions and its regulated secretion from monocytes by the inflammatory mediator CD40 ligand.[26] In addition, serine proteases can activate MMPs secreted from SMCs and degrade fibrin within advanced plaques.[27,28] Thus, different families of proteolytic enzymes may participate in atherogenesis.

Cathepsins of the cysteine protease family localize in lysosomes and endosomes and function there to degrade unwanted intracellular or endocytosed proteins. In the early 1990s, we recognized few of these proteases and knew little of their physiologic or pathologic functions. More recently, modern molecular biology permitted the characterization of a series of novel cathepsins, many widely expressed in humans and animals. However, the recognition of inducible cathepsins such as S, K, C, V, and W led to the unraveling of their roles in inflammatory and/or autoimmune diseases such as cancer, rheumatoid arthritis, osteoporosis, hyperkeratosis/periodontitis, and atherosclerosis.[29] Most strikingly, we now recognize that these lysosomal cysteine proteases can function outside lysosomes or endosomes, although these intracellular organelles still contain the bulk of these enzymes. Detection of active cysteine proteases in culture media of SMCs, ECs, and macrophages significantly broadened our understanding of their potential roles in arterial pathobiology. This review will discuss some of the recent findings in this field and highlight the significance of lysosomal cysteine proteases in arterial ECM remodeling and atherogenesis.

Regulated Expression of Cysteine Proteases and Their Endogenous Inhibitor, Cystatin C

Overexpression of Lysosomal Cysteine Proteases and Deficiency of Cystatin C in Atherosclerosis

Atherosclerotic lesions contain much more cathepsin S and K mRNAs and proteins than do normal arteries. Either protease can degrade elastin and collagen.[29,30] Cathepsin K, one of the most potent mammalian collagenases, participates in joint and bone collagen metabolism. Deficiency of this protease impairs bone growth in both humans and animals.[31,32] Immunohistochemical studies demonstrated expression of cathepsins S and K mainly in macrophages in the shoulder regions of atheromata, in SMCs of the fibrous cap, and at sites of internal elastic laminae fragmentation.[33] These findings suggest involvement of these proteases in degradation of elastic laminae in the vascular wall, which may facilitate SMC migration, and destabilization of atherosclerotic plaque by degrading collagen of the fibrous caps.[34] Also, the ECs lining the lumen of the vessel (not shown) as well as those in the plaque microvessels (Figure 1) express cathepsin S, indicating a role for this protease in neovascularization, a process implicated in plaque growth and complication.[35] In contrast, healthy human aortae contain no immunoreactive cathepsin S or K, although Northern blot analyses of healthy aortas have shown low levels of cathepsin K mRNA.[33] Western blot analysis and elastase assay of tissue extracts from human atherosclerotic lesions demonstrated increased levels of active forms of cathepsins S and K with significantly elevated elastolytic activity, sensitive to the cysteine protease inhibitor E64d or the cathepsin S selective inhibitor morpholinurea leucine-homophenylalanine-vinylsulfone-phenyl (LHVS),[33] suggesting an important contribution of cysteine proteases in arterial elastic tissue remodeling.

Atherosclerotic lesions in Apo E–deficient mice showed a similar increase in expression of lysosomal cathepsins S, L, and B.[36] As described in human atherosclerotic lesions,[33] cathepsin S localized mainly in intimal SMCs and macrophages and medial SMCs. Nonatherosclerotic arteries showed no expression of cathepsin S.[36] This expression pattern affirms an association of these proteases with atherogenesis in humans. Consistent with this hypothesis, human atherosclerotic lesions have relatively low levels of cystatin C, an endogenous inhibitor of these cathepsins, whereas normal arteries express abundant cystatin C in medial SMCs and ECs.[37] This expression profile suggests an imbalance between cysteine proteases and their inhibitors that favors arterial ECM breakdown. Similar loss of this counterbalance was observed in atherosclerotic lesions from Apo E–deficient mice. In mouse aortic atherosclerotic lesions, immunostaining for cystatin C was also much less intense than that in normal mouse aortas (Sukhova and Shi, unpublished data). Such inverse regulation of vascular proteases and protease inhibitors appears much less prominent in the case of MMPs and their tissue inhibitors (TIMPs). In human atherosclerotic lesions, TIMP expression changes little or increases compared with that in control vessels.[38–40] Murine atherosclerotic lesion development showed similar expression patterns.[36] Increased MMP-9 expression in Apo E–deficient, murine atherosclerotic lesions accompanies increased TIMP-1 levels, whereas expression of cysteine proteases and their inhibitor cystatin C remains inversely regulated.

Expression and Secretion of Lysosomal Cysteine Proteases and Cystatin C In Vitro

Macrophages, SMCs, and ECs account for most of the cysteine protease expression in human atherosclerotic arteries. To study the regulation of cysteine protease expression in these cells, we isolated human monocytes, vascular SMCs, and vascular ECs and then examined cysteine protease expression under different conditions. Although human blood monocytes express negligible levels of cathepsin K, maturation of monocytes into macrophages during incubation in 40% fetal calf serum markedly augments cathepsin K expression and elastolytic activity.[41] These human monocyte-derived macrophages also express and secrete cathepsins S, L, and B.[42,43] Similar to medial SMCs in the normal vessel wall, cultured SMCs do not express cathepsin S or K under basal conditions. However, incubation of these cells with inflammatory cytokines such as interleukin-1β (10 ng/mL), tumor necrosis factor-α (10 ng/mL), and interferon (IFN)-γ (400 U/mL) for 24 hours significantly induces the expression and secretion of cathepsins S and K at both the mRNA and protein levels.[33] Furthermore, increased cathepsin expression occurs in tandem with enhanced elastase activities, largely sensitive to E64, establishing a predominant contribution to the elastolytic activity of cysteine proteases in inflamed SMCs. More important, culture media from IFN-γ–stimulated SMCs contained active cathepsin S detected by both active-site labeling of de novo synthesized enzymes and by degradation of water-insoluble elastin.[33] These findings indicate release of cathepsin S from SMCs and interaction with ECM proteins, a process that likely occurs in atherosclerotic lesion development during SMC migration and further neointima formation.

Effect of Cytokines and Angiogenic Factors on Cathepsin S and Cystatin C Expression and Secretion

	Cathepsin S		Cystatin C	
	Expression	Secretion	Expression	Secretion
IFN-γ	↑ (SMC,ED)	↑ (SMC,EC,MΦ)	—	—
IL-1β	↑ (SMC,EC)	↑ (SMC,EC)	—	—
TNF-α	↑ (SMC,EC)	↑ (SMC,EC,MΦ)	↓ (EC)	↓ (EC)
TGF-β1	—	—	—	↑ (SMC)
VEGF	↑ (EC)	↑ (EC)	—	—
bFGF	↑ (EC)	↑ (EC)	↓ (EC)	↓ (EC)

↑ indicates increased; ↓ indicates decreased; — indicates no effect SMC, smooth muscle cells; EC, Endothelial cells; MΦ, macrophages.

In contrast, cystatin C, the endogenous inhibitor of the cysteine proteases, shows distinct regulation by different cytokines.[37] For example, IFN-γ or interleukin-1β increases cathepsin S expression 10- to 20-fold in SMCs but does not affect cystatin C expression. Tumor necrosis factor-α induces cathepsin S expression in SMCs but reduces cystatin C expression in vascular ECs.[37] We obtained similar results with the angiogenic stimulus basic fibroblast growth factor, which augments cathepsin S expression by >10-fold in ECs but also reduces cystatin C expression.[35] Grainger et al[44] detected significantly lower blood levels of transforming growth factor-β1 in patients with atherosclerosis, but transforming growth factor-β1 increases SMC cystatin C secretion by a posttranscriptional mechanism.[37] The Table lists the effects of various cytokines and angiogenic factors on cathepsin S and cystatin C expression and secretion in these cells. These observations indicate that some inflammatory factors differentially regulate the expression and secretion of lysosomal cysteine proteases and their endogenous inhibitors in arterial cells.

Proteolytic Activities of Extracellular Lysosomal Cysteine Proteases

Lysosomal cysteine proteases are synthesized and targeted to the acidic compartments, lysosomes and endosomes, via either the mannose-6-phosphate receptor–dependent pathway[45,46] or the mannose-6-phosphate receptor–independent pathway,[47] where they are activated to degrade their substrates. These compartments provide cysteine protease cathepsins with the optimal pH for their activity.[29] Although cathepsin cDNA sequences do not appear to encode a conventional secretory signal peptide,[48] several groups including our own have demonstrated the existence and activity (collagenolytic and/or elastolytic) of these proteases in media conditioned by SMCs, ECs, and monocyte-derived macrophages.[33,37,42,43] Therefore, several questions remain. These include how these proteases are released and how they retain

their activity once they have left the cells, because most of these proteases have a very narrow pH optimum (pH 4 to 6). For instance, cathepsins K and L lose their activity at neutral pH.[49] Although cathepsin S does exceptionally retain some activity at neutral pH,[50] it is questionable whether this partial activity explains all of the cysteine protease–dependent ECM degradation observed in vitro. Hence, in the recent proposal of a "focal contact" hypothesis, Punturieri et al[43] used human monocyte-derived macrophages to demonstrate the formation of a localized acidic environment in a zone of contact that excludes the surrounding extracellular milieux. Such focal contact permits lysosomal or endosomal cysteine proteases to degrade extracellular elastin efficiently. After incubating cultures for 24 hours, they detected an acidic interface between the cell surface and elastin filaments by use of a fluorescent pH indicator. Punturieri and colleagues further demonstrated by use of bafilomycin, an inhibitor of acidification of both intracellular and extracellular compartments that inhibited monocyte-derived macrophage elastase activity, that these extracellular acidic milieux resulted from increased expression of vacuole-type H^+-ATPase. These findings provide a reasonable explanation for ECM degradation by cysteine proteases released from macrophages.

Monocyte adhesion, migration, and differentiation into macrophages play essential roles in the pathogenesis of the atherosclerotic lesion. These cells may also use extracellular cysteine proteases to assist their migration during atherogenesis. We recently demonstrated in vitro that cathepsin S–deficient monocytes cannot migrate through artificial membranes containing SMCs, collagen mixtures, and an endothelial monolayer.[51] This finding suggests a critical involvement of cysteine proteases in blood-borne cell transmigration from peripheral blood into the vessel wall. Therefore, it is reasonable to hypothesize that these monocytes/macrophages may degrade ECM via the pathways of vacuole-type H^+-ATPase expression, extracellular acidic milieu formation, and extracellular collagen degradation. Validation of this hypothesis will require further investigation.

It is therefore conceivable that SMCs and ECs use the same focal contact mechanism to dissolve ECM as do macrophages.[33,35] Medial SMCs contain immunoreactive cathepsins S, K, and L in atherosclerotic human and animal vessel walls, notably near sites of internal elastic lamina fragmentation[33,51] (see below). These SMCs likely also form an extracellular acidic microenvironment that allows extracellular cathepsin S and possibly other elastolytic cathepsins (eg, K and L) to break down the elastic barriers. Several lines of evidence support this hypothesis. Stimulation of cultured human or murine SMCs with IFN-γ induced expression of elastolytic cathepsins and increased extracellular degradation of water-insoluble elastin. The cathepsin inhibitor E64d blocks much of this activity.[33,51] Furthermore, SMCs lacking cathepsin S showed a significantly reduced ability to degrade extracellular elastic fibers. Although we do not have direct evidence of formation of a cell-substrate acidic interface, we observed attachment of SMCs to exogenous water-insoluble elastin fibers in culture (Figure 2). Nevertheless, it remains to be confirmed whether SMCs act as monocyte-derived macrophages do and form acidic focal contact compartments that facilitate degradation of extracellular elastin in culture and within human and animal vascular walls.

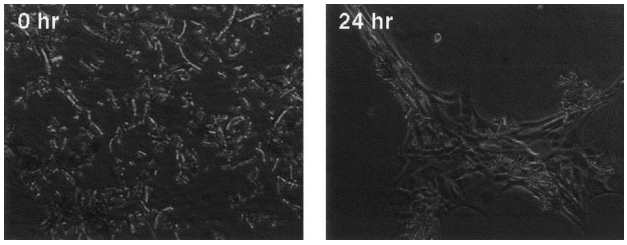

Figure 2. Focal contact of SMCs and elastin fibers. Human saphenous vein SMCs were cultured in monolayers. Water-insoluble elastin fibers were seeded on top of the cell monolayer (left). By 24 hours of culture, SMCs had formed direct focal contacts with elastin fibers (right).

Lysosomal Cysteine Proteases and Atherogenesis

Increased expression of cysteine proteases cathepsins S, K, B, H, and L and decreased expression of their endogenous inhibitor cystatin C in human atherosclerotic lesions suggested the involvement of cysteine proteases in atherogenesis. Recent findings from in vitro and in vivo experiments indicate that these cysteine proteases may participate in the main stages of atherogenesis.

Initiation

Atherogenesis initially involves leukocyte recruitment from the circulation by adhesion to the endothelium, followed by penetration through the endothelial layer and arterial basement membrane.[8] Current understanding implicates specific adhesion molecules expressed on the surface of vascular ECs, eg, vascular cell adhesion molecule-1 (VCAM-1), and chemoattractant molecules, such as macrophage chemoattractant protein-1 (MCP-1), in this process.[8] Deficiency or impaired function of these molecules significantly reduces atherogenesis in animals.[52–54] We currently do not know whether cysteine proteases play any role in regulating MCP-1 or VCAM-1 expression or leukocyte adhesion. Data from studies of cathepsin S–knockout mice illustrated a significant reduction of these molecules in sera of mice initiated for atherosclerosis with a high-cholesterol diet.[51] Therefore, cathepsin S may act like MMPs and release adhesion molecules from the surface of ECs.[55] Alternatively, cathepsin S may indirectly influence the production of adhesion molecules by affecting the γδ-T lymphocyte population,[56] another possible source of VCAM production.[57] On the other hand, the reduced levels of MCP-1 and VCAM-1 observed in cathepsin S–deficient mice may result from lower serum lipid levels[58] or decreased lesional monocyte content[59] and may not directly depend on cathepsin S activity.

After adhesion and transmigration through the endothelial layer and basement membrane, monocytes become macrophages, proliferate, and become lipid-laden foam cells. The arterial subendothelial basement membrane contains type IV collagen, laminin, and fibronectin.[60,61] Migration of blood-borne leukocytes requires degradation of these ECM components, as does microvessel ingrowth from vasa vasorum of the

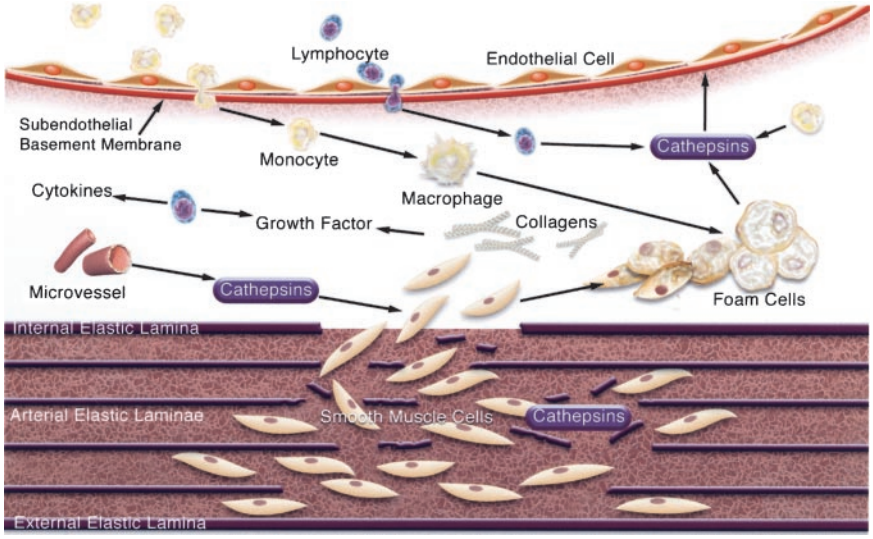

Figure 3. Potential roles of cysteine protease cathepsins in atherogenesis. The diagram depicts a cross section of an atherosclerotic lesion. Immunohistologic analysis demonstrated localization of cysteine protease cathepsins in the subendothelium (regulating blood-borne leukocyte transmigration), in SMCs in the fibrous cap (influencing plaque stability), at sites of internal elastic lamina fragmentation (controlling SMC migration), in macrophages and foam cells at the lipid core (degrading matrix proteins and affecting cell apoptosis), and in ECs of the vessel wall and microvessels (involving neovascularization). Inflammatory cytokines released from infiltrated lymphocytes, growth factors from degradation of matrix protein (eg, collagens), or even oxLDL may regulate cathepsin expression during atherogenesis. Both macrophages and SMCs take up modified LDL and become foam cells, which become the major components of the lipid core.

adventitia and tunica media.[62,63] Macrophages derived from human monocytes express and secrete substantial amounts of active cathepsins K, L, and S, which can degrade these subendothelial basement membrane components.[64–67] The mononuclear cells may well use these proteolytic enzymes to aid their transmigration. Indeed, the subendothelial basement membrane in early atherosclerotic lesions (fatty streaks) contains large amounts of immunoreactive cathepsins S and K.[33] To test for a role for cysteine proteases in monocyte transmigration, we recently used an in vitro preparation comprising layers of SMCs and a mixture of collagen types I and IV overlaid by a monolayer of ECs.[51] Human monocytes seeded on top of this structure migrated through the collagen matrix into the SMC layer, where they matured into macrophages. Interestingly, treatment of human monocytes with a low concentration (8 nmol/L) of LHVS (a condition that selectively inhibits cathepsin S) or a high concentration (1 μmol/L) of LHVS (that also inhibits other cysteine proteases) significantly reduced monocyte translocation (Shi et al, unpublished data). Cathepsin S–null monocytes yielded similar results.[51] More than 90% of cathepsin S–null monocytes remained atop the endothelial monolayer, whereas the majority of wild-type monocytes migrated into the SMC layer. These observations suggest involvement of cathepsin S and possibly other cysteine proteases in leukocyte transmigration.

Progression

During the progression of atherosclerosis, lipid-laden SMCs and macrophages accumulate along with T lymphocytes. These cells release inflammatory cytokines, and SMCs in particular elaborate ECM constituents. In addition to the transmigration of leukocytes from the lumen, SMC migration from the media also occurs. Medial SMC migration and neointimal formation are part of the main pathologic events during progression of atherosclerosis. This SMC migration requires traversal of the internal elastic laminas. Although the elastic laminae may contain fenestrae, dissolution of these elastic barriers may aid the migration of SMCs to the intima. In vitro experiments demonstrated that elastin peptides in a modified Boyden chamber halted aortic SMC migration.[68] Furthermore, in SMCs, intact elastin regulates actin stress fiber organization, proliferation, and migration via a heterotrimeric G protein–coupled pathway.[69] Thus, understanding atherogenesis requires identification of the proteases that mediate elastolysis. The ability of human monocyte-derived macrophages to release elastolytic cysteine proteases led us to hypothesize that SMCs may also use cysteine proteases for their migration through arterial elastin. We validated this hypothesis by showing reduced elastolytic activity in experiments in vitro with cysteine protease inhibitors with human SMCs and aortic SMCs isolated from cathepsin S–deficient mice.[33,51] In vivo experiments further supported this hypothesis: studies of atherosclerosis-prone LDL receptor–deficient mice that consumed an atherogenic diet for 8, 12, and 26 weeks showed that SMCs at sites of elastin fragmentation expressed cathepsin S similar to those in human lesions. Mice lacking both the LDL receptor and cathepsin S on the same diet showed substantially less fragmentation of the arterial internal elastic lamina, reduced intimal SMC accumulation and collagen production, and significantly smaller atherosclerotic lesions. Indirect evidence comes from recent observations of Apo E–deficient mice. In these atherosclerosis-prone mice, deficiency of the endogenous cysteine protease inhibitor cystatin C increased aortic elastolysis mediated by SMC cysteine protease and consequently enhanced medial degradation of elastic laminae (Shi and Sukhova, manuscript in preparation).

Oxidized LDL (oxLDL) may favor formation of foam cells and necrotic or apoptotic lipid cores, hallmarks of both human and murine atherogenesis. Several experiments have implicated cysteine proteases in these pathologic processes. The lysosomal cysteine proteases cathepsins B and L may process caspases and induce apoptosis[70–72] and localize in the cytoplasm and nuclei of apoptotic (caspase-3–positive) macrophages in human atheromata.[73] Inhibition of cathepsins B and L with E64 or Z-Phe-Arg-FMK protects mononuclear cells from oxysterol-induced cell death. Thus, cytotoxic oxLDL and associated oxysterols in atheromata may cause lysosomal destabilization and release of cathepsins, fostering

the apoptotic death of phagocytic cells and contributing to further atherosclerotic lesion evolution, including lipid core formation.[73,74] This proapoptotic mechanism may also apply to other cells in atheromata, such as SMCs and ECs. In cathepsin S–deficient, LDL receptor–null atherosclerotic mice, we detected not only significantly less accumulation of lipid in lesions and lower serum levels of LDL or total cholesterol[51] but also lower titers of autoantibody to both malondialdehyde-oxLDL and copper-oxLDL epitopes (Shi et al, unpublished data). Because some proteases have been linked to major histocompatibility class II–associated antigen processing and presentation, cathepsin S, and possibly other cathepsins, may participate in the adaptive humoral and cellular immune responses that operate during atherosclerosis.[75–77]

Neovascularization provides a portal for leukocyte trafficking[6] and entry of plasma constituents, including lipoproteins, and occurs during progression of atherosclerotic lesions.[78] This process requires lysis of the ECM to pave the way for neovessel formation.[79] Although the microvessels of various human malignant tumors express cysteine proteases, direct evidence for involvement of these enzymes in neovascularization emerged only recently from studies of cathepsin S–deficient mice.[35] Healing skin wounds in cathepsin S–null mice contained far fewer microvessels. Defining the contribution of impaired neovascularization due to loss of cathepsin S activity to reduced atherosclerosis in cathepsin S–null mice is difficult. However, in human atherosclerotic lesions, areas of microvascularization contain both cathepsin S and its inhibitor cystatin C (Figure 1), in contrast to other regions of plaque that contain less cystatin C.[37] Therefore, cathepsin S and possibly other cysteine proteases may also regulate the growth of lesion microvessels during atherogenesis.

Complications

Thrombotic complications of atherosclerosis often involve plaque rupture. Lesions that have ruptured typically have a thin fibrous cap, prominent lipid deposition, and macrophage accumulation. Such thrombotic complications actually cause most of the acute manifestations of atherosclerosis, such as myocardial infarction or stroke.[7,8] Indeed, thrombus formation usually results from physical disruption of atherosclerotic plaque and appears related to the level of collagen in the lesion's fibrous cap. Therefore, the collagen content of the fibrous cap critically influences plaque stability and may depend in turn on the expression of interstitial collagen-degrading proteases.

Vulnerable human atherosclerotic plaques overexpress several MMP collagenases capable of degrading interstitial collagen types I and III.[79] For example, macrophages in plaques contain abundant MMP-1, -8, and -13 and colocalize with sites of collagen degradation in situ. Macrophages appear to furnish the bulk of cysteine proteases in atheromata. Indeed, Chen et al[80] used near-infrared imaging to detect cathepsin B activity in vivo in atheromatous lesions of Apo E–deficient mice. Increased expression of cathepsin B in atheromatous plaques colocalized with the macrophage marker Mac-3. We made similar observations in sections of human atherosclerotic lesions with cathepsins S, K, and L, all of which can degrade collagen.[29,30] Cathepsins S and K were originally isolated from human macrophages, and inflammatory cytokines increase cathepsin S secretion from macrophages (the Table).[81] In human atherosclerotic plaques, macrophages and fibrous cap SMCs express all 3 collagenolytic cathepsins[33] (Shi, unpublished data). However, the possible involvement of cysteine proteases in plaque rupture requires further examination. Fibrous cap thickness is directly associated with plaque vulnerability. Cathepsin S/LDL receptor double-deficient mice have decreased SMC and collagen contents in the lesions and a reduction in the size of the fibrous cap compared with those in control LDL receptor–null mice. Such a reduction in fibrous cap size could be the result of a decrease in the number of SMCs in lesions, which may increase plaque vulnerability.[34] Cystatin C/Apo E double-deficient mice consistently had increased lesional SMC and collagen contents and better developed fibrous caps (Sukhova et al, unpublished data), characteristics of stable plaques in humans. Thus, cysteine proteases could play dual roles. Medial SMCs release cathepsins to allow them to pass through the elastica and accumulate in the neointima, where they produce collagens and reinforce the fibrous caps.[34] On the other hand, SMCs in the fibrous cap can also produce cathepsins,[33] which are collagenolytic.[29,30] These cathepsins may cause weakening of the fibrous cap and lead to plaque rupture, although this conjecture requires experimental validation.

To date, no direct experiment has tested for a role of cysteine proteases in thrombosis during atherogenic complications. Data from our laboratory and collaborators indicate that cathepsin S regulates thrombotic responses to arterial injury.[82] After photochemical carotid artery injury, the time to the development of occlusive thrombosis decreased in cathepsin S–null mice. The accelerated thrombotic response to arterial injury and the shortening of plasma clotting times accompanied an increased activity of coagulation factors VIII, IX, and X and plasma von Willebrand factor, as measured by 1-stage clotting-based assays in cathepsin S–deficient mice. These results suggest that cathepsin S has antithrombotic properties. However, the mechanism by which cathepsin S affects thrombosis and whether its antithrombotic properties impact atherogenesis remain undetermined.

Conclusions

Numerous observations now support key functions of cysteine protease cathepsins in atherogenesis (Figure 3). Cathepsins may pave the way for entry of blood-borne monocytes and lymphocytes into the arterial vessel wall by degrading the subendothelial basement membrane and for the migration of SMCs through elastic laminae to enter the intima. Several cell types, including medial SMCs, neointimal SMCs, macrophages, and neointimal microvascular ECs, express cathepsins in atheromatous lesions. Exposure to inflammatory cytokines or growth factors released by transmigrated monocytes, T lymphocytes, or ECM degradation products may enhance the expression or release of cathepsins in these cells (Figure 3 and the Table), a hypothesis supported by observations from studies of cultured cells. Thus, the inflammatory process at the core of atherogenesis is linked tightly with proteolysis due to cathepsins. Furthermore, cathepsin func-

tion in thrombosis[82] or even immunity[76,83] also may contribute to the pathogenesis of atherosclerosis, although we lack direct evidence for these functions. Several recent reports related primarily to the remodeling of the ECM do establish an important role for cysteine proteases in atherogenesis in genetically altered mice. We therefore propose that multiple proteases, including MMPs and serine proteases, work in concert during the initiation, progression, and complication of atherosclerotic plaques.

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