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Cystatin C and cathepsins in cardiovascular disease

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1. ABSTRACT

Cystatin C and cathepsins could play a role in almost all processes involved in atherosclerotic lesion formation by their degradation of extracellular matrix proteins and apolipoprotein B100, the protein moiety of LDL. Several cysteine cathepsins are upregulated in human lesions accompanied by a decrease in cystatin C, the major inhibitor of cysteine cathepsins. Recent research show that atherosclerotic mice deficient in cystatin C display increased elastic lamina degradation as well as larger plaque formation. Cathepsin S- and K-deficient atherosclerotic mice, on the other hand, both have less atherosclerosis, where cathepsin S-/- mice exhibited fewer plaque ruptures and cathepsin K-/- larger foam cells than control mice. This article reviews possible roles of cystatin C and cathepsins in different processes and stages of the atherosclerotic disease.

2. INTRODUCTION

Atherosclerosis is a chronic inflammatory disease affecting the intima of medium- and large arteries. It is responsible for the development of most acute cardiovascular events including myocardial infarction and stroke. It is generally believed to be activated by an accumulation of low density lipoprotein (LDL)-derived lipids in the vascular wall extracellular matrix (ECM). This accumulation is mediated by binding of the LDL protein apolipoprotein B-100 (apoB-100) to ECM components such as the negatively charged glycosaminoglycan (GAG) chains of proteoglycans (1). LDL particles entrapped in the ECM tend to aggregate and become oxidized. The oxidized LDL triggers an inflammatory response including expression of adhesion molecules and leukocyte infiltration. Uptake of modified LDL by monocyte-derived macrophages results in the formation of foam cells.

Chronic intimal inflammation results in activation of vascular repair responses involving migration of smooth muscle cells (SMC) from the media to the intima where they proliferate and synthesize ECM proteins. At more advanced stages these SMC form a fibrous cap covering a lipid-filled, inflammatory and necrotic core. Degradation of the fibrous cap increases the risk for plaque rupture and development of acute cardiovascular events. These ruptures occur primarily in the shoulder regions of the lesions, and are believed to be caused by macrophages secreting proteases resulting in degradation of ECM proteins, in combination with a decreased proliferative capacity of injured or apoptotic SMC (2). Proteases and their inhibitors are likely to play important roles at all stages of the disease by affecting cell adhesion, migration, proliferation, plaque rupture and induction of apoptosis. The proteases found to be present in lesions include matrix metalloproteinases (MMPs), serine proteases and cathepsins (3, 4). There has been much attention on the role of MMPs in plaque development and rupture. However, during recent years it has become clear that also cathepsins play important roles in these processes (5). Cystatin C, the main inhibitor of the cysteine family of the cathepsins, is produced and secreted by most cells. The cysteine family of cathepsins are lysosomal proteases, synthesized as preproenzymes, which are activated upon cleavage in the acidic environment of late endosomes or lysosomes (6, 7). Although they have an acidic pH optimum, secretion of active cathepsins as well as activity at neutral pH has been reported (8, 9). The cathepsins are known to degrade elastin and collagens, the two major ECM constituents of the vessel wall. As much as 60% of the elastolytic activity of macrophages has been attributed to cysteine proteases (10). Moreover, other matrix components such as osteopontin, osteonectin, proteoglycans, tenascin-C, fibronectin, the basement membrane proteins laminin and collagen type IV as well as apoB-100 are substrates for different cysteine cathepsins (11-16). In addition, cathepsins, as well as cystatin C, have been implied to play a role in antigen presentation (17). The latter may be of importance in atherosclerosis since immune responses against modified apo B-100 antigens have been associated with disease development in humans (18). The cathepsin cysteine proteases known today are the cathepsins B, H, L, S, C, K, O, F, V, X, and W (19). Cystatin C has been shown to inhibit cathepsins K, B, H, L and S (20, 21) and to bind to cathepsin F (12).

3. CYSTATIN C AND CATHEPSINS IN ATHEROSCLEROTIC LESIONS

Cystatin C is expressed in vascular SMC and found in normal arteries, but is severely reduced in atherosclerotic lesions (22). Several cathepsins (S, K, B, L, H, V, F, and W), on the other hand, are found in human atherosclerotic lesions (10, 12, 23-28). The cathepsins are produced by macrophages or cytokine-stimulated SMC, as well as endothelial cells (23, 27-29). These data suggests an altered balance of cystatin C versus cathepsins in atherosclerotic lesions compared to unaffected vessels. The cathepsins are increased in atherosclerotic lesions of mice (30, 31). mRNA levels of cathepsin B, L, and S were upregulated in aortas of atherosclerotic apolipoprotein E-deficient (apoE-/-) mice as compared to healthy C57BL/6 mice, whereas mRNA levels of cystatin C did not differ (31). The extracellular cystatin C content in lesions may be regulated at the translational or secretional rather than the transcriptional level, since neither IFNγ nor TGFβ altered mRNA levels of cystatin C in SMC, but TGFβ stimulated SMC secretion of cystatin C. However, cystatin C does not seem to be completely downregulated in lesions, since cystatin C was detected in atherosclerotic lesions of apoE-/- mice by immunohistochemistry (32) and low levels of the protein was present in extracts of human lesions by Western blot (22).

4. CYSTATIN C- AND CATHEPSIN-DEFICIENT MOUSE MODELS AND ATHEROSCLEROSIS

The availability of knock-out mouse models of cystatin C and cathepsins have made it possible to study the role of these proteases and their inhibitor in atherosclerosis *in vivo*. These studies point to a role of cystatin C as an anti-atherogenic protein, protecting against enhanced elastin degradation. Atherosclerotic apoE-/- mice deficient in cystatin C developed larger lesions compared to cystatin C expressing apoE-/- mice and this atheroprotective effect was shown to be due to cystatin C produced by nonhematopoietic cells, since apoE-/- mice transplanted with cystatin C- and apoE-deficient bone marrow cells did not show any differences in lesion area (33). It has also been shown that apoE-/- mice lacking cystatin C have increased elastic lamina degradation and aortic dilatation with an expanded abdominal aorta (34). Cathepsins on the other hand seem to be mostly proatherogenic. LDL receptor-deficient mice (LDLR-/-) mice crossed with cathepsin S-deficient mice had less atherosclerosis, with less macrophages, lipids, SMC, collagen and CD4+ T-lymphocytes, compared to mice deficient in only LDLR-/- (35). Cathepsin S-deficient apoE-/- mice displayed smaller lesion areas compared to control mice (26). Cathepsin K was the first cathepsin reported to cleave the triple helical region of collagen (36, 37). Although both cathepsin S and L display some triple helical collagenolytical activity, cathepsin K has the highest activity (38). Accordingly, apoE-/- mice lacking cathepsin K have smaller lesions with more collagen, but also less medial elastic degradation (27).

5. CYSTATIN C AND CATHEPSINS IN ATHEROSLCEROTIC LESION FORMATION AND PROGRESSION

5.1. LDL-retention and LDL-modification/oxidation

A key step in atherosclerotic lesion formation is retention of LDL in the ECM of the vessel wall followed by aggregation and modification/oxidation of the LDL particles. The most studied interaction between LDL and the ECM is that of negatively charged GAG chains of proteoglycans with positive amino acid residues in apoB-100, but also collagen and elastin has been shown to bind LDL (39). The cathepsins may play a role in this process, as it was shown that cathepsin F cleaves apoB-100, triggering aggregation and fusion of LDL particles and enhanced binding of LDL to human arterial proteoglycans *in vitro* (12). Furthermore cathepsins B, F, K, L, S and V

were all demonstrated to cleave apoB-100, which resulted in enlarged LDL particles (15).

5.2. Monocyte infiltration, differentiation and macrophage foam cell formation

Oxidized LDL initiate an inflammatory response, attracting monocytes and T-lymphocytes to the vessel wall. The monocytes differentiate into macrophages, and the uptake of oxidized LDL mediated by scavenger receptors results in lipid-rich macrophage foam cells. The infiltration of monocytes requires degradation of the subendothelial basement membrane proteins as well as of intimal matrix molecules by proteases. Indeed, LDLR-/- cathepsin S-deficient mice displayed reduced macrophage content, and the corresponding macrophages displayed decreased subendothelial basement membrane transmigration (35). Cathepsin K, on the other hand, does not seem to play an important role in monocyte infiltration of the lesion, since macrophages from atherosclerotic mice deficient in cathepsin K did not show an altered migration in a Matrigel matrix assay, a reconstituted basement membrane *in vitro*, neither did the relative macrophage content of the lesions differ (27). Differentiation of monocytes to macrophages with IL-3 induced expression of cathepsin K and V (10). Cathepsin expression may also increase during foam cell formation as shown by enhanced cathepsin S expression in human macrophages in response to oxidized-LDL treatment (16). Furthermore, LDL modified by cathepsin H and cholesterylesterase conferred LDL the capacity to induce macrophage foam cell formation and at the same time induced cathepsin H expression, implying a contribution of cathepsin H in the process of transforming LDL to atherogenic LDL (25). A possible role for cathepsin S in macrophage differentiation and/or foam cell formation was also reported by Sukhova *et al*, describing smaller monocytes/macrophages in cathepsin S-/- LDLR-/- mice compared to LDLR-/- mice (35). Further evidence for a role of cathepsins in foam cell formation *in vivo* was presented by Lutgens *et al*, who saw an increased individual macrophage size in atherosclerotic cathepsin K-deficient mice and increased scavenger receptor mediated uptake of LDL in macrophages from cathepsin K deficient mice compared to control mice (27). Also in the cholesterol efflux from macrophage foam cells, cathepsins may be of importance, since this was blocked by cathepsin F and S (40).

5.3. SMC migration, proliferation and fibrous cap formation

When the atherosclerotic lesions progress, SMC migrate from the medial layer of the vessel wall to the intima and switch from a contractile phenotype to a synthetic phenotype, producing collagen. SMC migration through the internal elastic lamina, separating the media from the intima, is proposed to occur through fine openings in the elastic lamina (41). However, it is possibly that an additional degradation of the elastin layer by proteases would facilitate SMC migration. Studies supporting a role for cathepsins and their inhibitor cystatin C in this process include both immunohistochemical analysis and knock-out mice. It was first reported that intimal SMC appearing to traverse the internal elastic lamina contained cathepsin S and K (23). In addition, atherosclerotic apoE-/- mice deficient in cystatin C display increased elastic lamina degradation and SMC content in lesions compared to apoE-/- mice (34). In line with these results, atherosclerotic mice deficient in cathepsin S or cathepsin K had preserved elastic laminas compared to control mice (27, 35). Recently, it was shown that cathepsin S is located at the surface of vascular SMC, and that cystatin C attenuated SMC invasion across an elastin gel. Immunofluorescence demonstrated a partial colocalization of cathepsin S and integrin $\alpha_v\beta_3$ (42).

5.4. Plaque stability and plaque rupture

The mechanisms responsible for development of plaque rupture remain to be fully elucidated, but are believed to involve degradation of the ECM by MMPs released by activated macrophages and impaired repair processes due to decreased SMC viability. Generally, lipid filled lesions with a thin fibrous cap are regarded more vulnerable than fibrotic plaques with a thick cap. Plaques usually rupture at the shoulder regions, therefore the presence of macrophages secreting proteases in the combination of lack of SMC producing ECM in these regions is probably of greater importance than the total plaque composition (2). A study of mice deficient in apoE and cathepsin S strongly supports that cathepsins may play a role in plaque stability. These mice displayed fewer acute plaque ruptures and less buried fibrous layers in their brachiocephalic arteries than control mice (26). The latter are believed to represent old healed ruptures. In addition, active cathepsins were mainly present in lipid-rich macrophages in the shoulder regions of human lesions. Moreover, cathepsin K deficient apoE-/- mice showed increased collagen content in advanced lesions, thus resulting in a more fibrotic, and possibly more stable, plaque phenotype (27).

5.5. Apoptosis

Apoptosis of macrophages in early lesions seems to limit plaque progression, whereas in advanced plaques, apoptosis may contribute to the inflammatory response and instability of the lesion (43). In human atherosclerotic lesions cathepsins B and L are co-localized with apoptotic macrophages. In addition, inhibitors of cathepsin B and L provided protection against oxysterol-induced apoptosis and reduced activation of caspase-3, suggesting a role for cathepsins as caspase activating enzymes (24).

5.6. Plaque regression

While intensive statin treatment has in general been implied to stabilize plaques rather than making them regress, animal models of plaque regression have been developed. One of these comprises the atherogenic apoE -/- mouse model bone-marrow transplanted with apoE-competent bone-marrow (44). In this setting absence of cystatin C in hematopoietic cells result in increased plaque area, and unexpectedly an increase in collagen and elastin content in the lesions (32). However, treatment of patients with modern highly potent statin has been able to show plaque regression making the plaque regression mechanism a clinical real-life phenomena (45).

6. AORTIC ANEURYSMS

Aortic aneurysms accumulate inflammatory cells, but in contrast to atherosclerotic lesions, the intraluminal diameter of aortic aneurysmal lesions initially often appears normal. Instead, a more extensive medial and adventitial inflammatory cell invasion and medial degradation of elastin and collagen take place (46). Accordingly, increased content of cathepsins as well MMPs have been shown in aneurysmal vessels. Gacko *et al* demonstrated higher content and activity of cathepsin B and L, but lower levels of cystatin C in aortic aneurysms than in normal aorta (47-49). Likewise, Liu *et al* (28) reported increased expression of cathepsin L and localized it to lesional SMC, endothelial cells and macrophages in human abdominal aortic aneurysms and Shi *et al* (22) reported reduced expression of cystatin C in aneurysmal tissue. In addition, serum levels of cystatin C was lower in serum from patients with dilated aorta compared to patients with normal aorta (22). Using DNA expression array technique, Tung *et al* reported a 30-fold increased expression of cathepsin H in abdominal aortic aneurysms compared to normal aorta (50).

7. NEOINTIMA FORMATION

Treatment of atherosclerotic lesions by balloon angioplasty with or without subsequent stenting often results in restenosis, i.e. a narrowing of the vessel lumen. The mechanisms of restenosis are believed to involve constrictive remodeling as well as increased SMC proliferation and fibrosis. The later phenomenon has been extensively studied in animal models of balloon or collar-induced vascular injury. Mechanical injury of a normal artery activates SMC migration from the tunica media to the intima leading to development of thickened intimal layer referred to as neointima. Like in the formation of the fibrous cap in atherosclerotic lesions, SMC migration during arterial remodeling in neointima formation requires degradation of the internal elastic lamina. In balloon injury of the carotid artery of rats, cathepsin S and K were upregulated as shown by RT-PCR and Western blot, whereas cystatin C levels remained unaltered (51). In addition the elastolytic activity of the injured vessel was increased due to cysteine protease activity. The collagenolytic activity was partly attributable to cysteine proteases and partly to MMPs. Burns-Kurtis *et al* also reported in increased cathepsin expression in balloon-injured hypercholesterolemic rabbits (16). In the study, cathepsin S was upregulated 28-fold in injured iliofemoral arteries compared to control, whereas cystatin C was upregulated to a lesser degree (3.5-fold).

8. ANGIOGENESIS

Angiogenesis has been proposed to contribute to plaque growth, leukocyte infiltration and plaque rupture (52). The angiogenic process involves degradation of the basement membrane underlying endothelial cells, but also of the matrix components of the intima. Cathepsin S, K, and L, as well as cystatin C are expressed in endothelial cells (27-29). However, their expression profiles in control cells compared to cells stimulated with inflammatory cytokines and angiogenic factors (bFGF and VEGF) differed (29). Cystatin C production decreased upon TNFα and bFGF-stimulation, whereas cathepsin S secretion was enhanced by IFNγ, VEGF and bFGF. *In vitro* studies showed that cystatin C inhibited EC tubule formation. In addition, cathepsin S deficient mice displayed reduced microvessel formation in wound healing of mouse skin (29). Cathepsin S-deficient mice also showed an impaired angiogenesis in a mouse model, which develop pancreatic islet cell carcinogenesis, whereas the cystatin C deficient mice displayed the opposite phenotype (53).

9. CATHEPSINS AND EXTRACELLULAR ACTIVITY

Since cathepsins were regarded as lysosomal proteases with an acidic pH optimum, proteases in cardiovascular disease were for a long time focused on MMPs. However, it has now been shown that cathepsins are secreted in their active form by monocyte-derived macrophages and cytokine-stimulated SMC in culture (9, 12, 23, 54). In addition, PDGF-BB-stimulated SMC invasion through elastin and collagen gels were inhibited by cystatin C (42). The elastolytic activity was almost completely attributed to cysteine cathepsins, whereas collagenolytic activity was primarily dependent on MMPs.

Most cysteine cathepsins, with cathepsin S as one exception, are labile at neutral pH. However, monocyte-derived macrophages can create a pericellular acidic environment where degradation takes place. Punturieri *et al* showed that macrophages generated an acidic milieu surrounding cell-bound elastic particles, in concert with an upregulation of vacuolar-type H+-ATPase components, which previously have been shown to be involved in acidification of intra- and extra-cellular compartments (9). In addition, Naghavi *et al* found that lipid-rich regions of the lesions had lower pH than calcified areas, and interestingly these low pH regions were associated with an increase in temperature, implying more inflammation (55). Another possibility by which cathepsins could display extracellular activity is that a constitutive secretion of active – though shortlived - cathepsins pericellularly would enable matrix degradation also at a neutral pH. It is also possible that part of the elastin degradation of cathepsins occurs by phagocytosis of insoluble elastin particles. By the use of cell permeable and non-cell permeable cysteine protease inhibitors, Yasuda *et al* showed that 2/3 of the cysteine protease elastolytic activity of cultured monocyte derived macrophages was attributed to extracellular activity and 1/3 to intracellular activity (10).

10. CYSTATIN C AND CATHEPSIN LEVELS IN THE CIRCULATION AND ASSOCIATIONS TO CARDIOVASCULAR DISEASE

Kidney dysfunction is associated with increased risk of death from cardiovascular causes (56). Cystatin C is a marker for kidney glomerular filtration rate and has been shown to be a stronger predictor of the risk of death and cardiovascular events in elderly persons than creatinine (57). Association of plasma levels of cystatin C with

symptomatic peripheral arterial disease has been studied by Albert *et al*, who found no association of cystatin C and future arterial disease (58). This relation might be hard to establish since the patients with an atherosclerotic burden should have a higher incidence of impaired blood flow to their kidney due to their atherosclerotic burden and thus a decrease in glomerular filtration rate would follow. Therefore a supposed decreased level in the circulation of cystatin C due to genetic influence, such as promoter polymorphisms, could be masked by an increase of cystatin C in the circulation due to impaired glomerular filtration rate. Still cystatin C promoter polymorphisms have been related to levels of cystatin C in the circulation and then to number of coronary stenoses per coronary segment (59). Other investigators found relations between cystatin C promoter polymorphisms and levels in the circulation, but could not relate them to clinical end-points such as fatal or non-fatal cardiovascular events (60).

Increased serum levels of both cathepsin S and L have been associated with atherosclerotic stenosis (28, 61). Furthermore, a strong correlation was seen between cathepsin L levels in serum and severity of coronary disease (28). Patients with acute myocardial infarction or unstable angina had higher levels of cathepsin S (61). Cathepsin S was also increased in patients with diabetes, who are associated with increased risk for cardiovascular diseases accompanied by increase elastolysis. Also cystatin C levels were higher in diabetic patients than in controls (61). Even if adjusted for creatinine, the higher cystatin C level in patients could still be related to a lower glomerular filtration rate, since cystatin C is a much more sensitive estimate of kidney function than creatinine which has a low sensitivity (62).

11. SUMMARY

In summary, several studies both *in vitro* and *in vivo* emphasize important roles for cystatin C and cathepsins in lesion initiation, progression and subsequent plaque rupture. Different cathepsins have been shown to have different roles, thus further *in vivo* research of the cathepsin family is required to fully understand their role in cardiovascular disease. Protease activity in atherosclerotic disease can be beneficial, e.g. matrix degradation necessary for SMC migration forming a protective fibrous cap, but could also have adverse effects, e.g. matrix degradation in plaque rupture, in the atherosclerotic disease. Even the same process may be beneficial at early time-points but aggravate the disease at later stages. Taking the complexity of the disease into account, it may be that both cathepsins and their inhibitor cystatin C, could exhibit both pro- and antiatherogenic properties, depending on which stage of the disease is studied. Further studies will be important to clarify the extra- versus intra-cellular activities of the cathepsins at different stages in the atherosclerotic process.

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Abbreviations: ECM, extracellular matrix; GAG, glycosaminoglycan; SMC, smooth muscle cells; MMPs, matrix metalloproteinases; apoB-100, apolipoprotein B-100; apoE-/-, apoE-deficient; LDLR-/-, LDL receptor-deficient.

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